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Mechanisms of Organophosphorus (OP) Injury: Sarin-Induced Hippocampal Gene Expression Changes and Pathway Perturbation

Victor T. Chan
Armando Soto
Jessica A. Wagner
Brandy S. Watts

Bioeffects Division
Molecular Bioeffects Branch

Amy D. Walters
Tiffany M. Hill

Henry M. Jackson Foundation for the Advancement of Military Medicine

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Air Force Research Laboratory
711th Human Performance Wing
Human Effectiveness Directorate
Bioeffects Division
Molecular Bioeffects Branch
Wright-Patterson AFB OH 45433-5707

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Armando Soto
Work Unit Manager
Molecular Bioeffects Branch
Bioeffects Division
Human Effectiveness Directorate
711th Human Performance Wing
Air Force Research Laboratory

Garrett D. Polhamus, DR-IV, DAF
Chief, Bioeffects Division
Human Effectiveness Directorate
711th Human Performance Wing
Air Force Research Laboratory

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14. ABSTRACT

This project aimed to investigate the molecular mechanism(s) of organ injury following exposure to sublethal doses of the organophosphorus agent, sarin. The lethal toxic effect of sarin exposure is caused by irreversible inhibition of acetylcholinesterase (AChE) and subsequently excessive stimulation of postsynaptic cholinergic receptors. However, much less is known about the effects of low-dose sarin exposure on functions of critical brain regions. Neurotoxicity caused by sarin exposure can be mediated by pathways independent of the cholinergic system. Since the hippocampus plays an important role in cognitive functions (including learning and memory), and is one of the most sensitive brain regions to sarin-induced injury, the focus of this study is to investigate differential gene expression and pathway perturbation in this brain region, using microarray technology combined with advanced bioinformatics. Sarin treatment resulted in differential expression of a large number of genes. Most of these genes showed increased expression, especially at the 6-hour and 25-day time points. However, significant gene repression was observed at the 10-day time point. Pathway analysis revealed that one of the direct effects of sarin cellular toxicity could be related to DNA damage and inhibition of the glutathione antioxidant system, which triggered the up-regulation of the apoptotic pathways at early time points.

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PREFACE

This research was conducted at the Molecular Bioeffects Branch (RHDJ), Human Effectiveness Directorate, 711th Human Performance Wing, Air Force Research Laboratory, Wright-Patterson AFB OH, under Dr. John J. Schlager, Branch Chief. This technical report was written as the Final Report for AFRL Work Unit ODTWP001. This project was funded by Defense Threats Reduction Agency (DTRA).

Employees of Henry M. Jackson Foundation (HJF) were working under the Cooperative Agreement FA8650-05-2-6518 between RHDJ and HJF. Animal exposure was conducted at Wright State University (WSU) under the HJF Cooperative Agreement FA8650-05-2-6518, Subaward # 667357.

All studies involving animals were approved by the WSU Institutional Animal Care and Use Committee (IACUC) on 11August 2009, and by the Wright-Patterson IACUC on 29 September 2009. All procedures involving live animals were conducted in a facility accredited by the Association for the Assessment and Accreditation of Laboratory Animal Care, International, in accordance with the *Guide for the Care and Use of Laboratory Animals*, National Research Council (1996). All experiments were conducted according to the Animal Use Protocol AUP #810 "*Organ Specific Perturbation of Biological Pathways and Processes Resulted from Exposure to Organophosphorus Agent in Mice (Mus musculus)*".

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SUMMARY

This project aimed to investigate the molecular mechanism(s) of organ injury following exposure to sublethal doses of the organophosphorus agent, sarin. The lethal toxic effect of sarin exposure is caused by irreversible inhibition of acetylcholinesterase (AChE) and subsequently excessive stimulation of postsynaptic cholinergic receptors. However, much less is known about the effects of low-dose sarin exposure on functions of critical brain regions. Neurotoxicity caused by sarin exposure can be mediated by pathways independent of the cholinergic system. Sarin exposure, even at non-convulsive and clinically asymptomatic doses, can induce prolonged adverse effects on cognitive and neurobehavioral functions in animal models. Consistent to these observations, sarin exposure resulted in cognitive deficits in the survivors of the sarin attacks in Japan. Rescue workers and victims, who did not develop any symptoms of acute cholinergic neurotoxicity, also exhibited chronic decline in memory. Since the hippocampus plays an important role in cognitive functions (including learning and memory), and is one of the most sensitive brain regions to sarin-induced injury, the focus of this study is to investigate differential gene expression and pathway perturbation in this brain region, using microarray technology combined with advanced bioinformatics. The results of functional observational battery (FOB) assessment revealed that sarin treatment, at 0.4x and 0.6x Mean Lethal Dose (LD50), were asymptomatic (or minimally symptomatic). However, ~60% of the animals treated with the dose of 0.75x LD50 had seizure. In contrast to the FOB result, sarin treatment, at 0.6x LD50, resulted in significant inhibition of AChE in the frontal lobe, especially at 2-6 hours after the treatment. As expected, a larger degree of AChE inhibition was observed with 0.75x LD50. Sarin treatment resulted in differential expression of a large number of genes. Most of these genes showed increased expression, especially at the 6-hour and 25-day time points. However, significant gene repression was observed at the 10-day time point. Pathway analysis revealed that one of the direct effects of sarin cellular toxicity could be related to DNA damage and inhibition of the glutathione antioxidant system, which triggered the up-regulation of the apoptotic pathways at early time points. To re-establish the homeostatic state, transcription, mRNA processing and splicing, translation and post-translation modifications were subsequently activated. The genes being up-regulated are involved in signaling pathways (both cell-cell signaling and intracellular signaling), cell proliferation (including neurogenesis), neuron differentiation, neuron projection development, axonogenesis, dendritic development, synaptogenesis, synaptic transmission, synaptic plasticity and behavioral/cognitive functions. However, processing of the newly synthesized proteins (such as protein folding and protein transport) appeared to be dysregulated, as the genes essential for components of the endomembrane system, including endoplasmic reticulum (ER) and Golgi apparatus were significantly down-regulated. As a result of dysfunction of endomembrane system, the ER stress response (including unfolded protein response and ER overload response) were up-regulated, which in turn induced the up-regulation of the apoptotic pathways at the subsequent time points. This finding is likely a participating event of the known sarin-induced delayed neurotoxicity.

Keywords: organophosphorus agent, sarin, acetylcholinesterase, DNA microarray, advanced bioinformatics, differential gene expression, pathway perturbation, unfolded protein response, endoplasmic reticulum (ER) overload, ER stress

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1. INTRODUCTION

1.1 Toxicity of Sarin Exposure

Sarin elicits its acute lethal toxic effects in large part by irreversible inhibition of AChE, resulting in excessive stimulation of postsynaptic cholinergic receptors. The clinical sequelae following high-dose sarin exposure include tremors, muscle fasciculation, salivation, seizure, respiratory depression, coma, and death. Animal studies have provided rich data on the acute toxic consequence and sequelae progression following high-dose exposure. However, much less is known about the effects, especially the long-term toxic effects, of low-dose sarin exposure on functions of critical brain regions and major peripheral organs. In addition to its cholinergic effects, there is evidence that neurotoxicity caused by sarin exposure can be mediated by pathways independent of the cholinergic system (Fernando et al, 1985; Rickett et al, 1986; Kadar et al, 1995).

In addition to its neurotoxic effects, sarin exposure may also result in injury of multiple vital organs, including heart, liver, kidney, bladder and adrenal gland via poorly understood mechanisms (Singer et al, 1987; Anzueto et al, 1990; Pant et al, 1993; Allon et al, 2005; Shih et al, 2006; Yanagisawa et al, 2006; Bloch-Shilderman & Levy, 2007; Morris et al, 2007). While sublethal doses of sarin do not result in acute overt toxicity, they have been shown to produce delayed damage and/or functional deficits in multiple vital organs. As exposure to low-level chemical warfare agents including sarin has become a world-wide emerging threat to military and civilian populations, it is therefore essential to elucidate the molecular mechanisms of sarin-induced organ injury.

Sarin exposure evidence has shown that even at non-convulsive and clinically asymptomatic dose, prolonged adverse effects can occur causing cognitive and neurobehavioral dysfunctions in a dose-dependent manner (Landauer et al, 1984; Kassa et al, 2001a; Kassa et al, 2001b; Kassa et al, 2001c; Kassa et al, 2002; Kassa et al, 2004; Genovese et al, 2008). A similar effect of sarin treatment on cognitive functions has also been observed in guinea pigs and nonhuman primates (Hulet et al, 2002; Genovese et al, 2007). Grauer et al (2008) reported that rats exposed to sarin resulted in long-lasting impairment of working and reference memory processes with no recovery observed during the study. Animals that seem to fully recover from the exposure, as well as the animals that initially show no toxic signs, developed neural changes indicative of neuronal inflammation lasting up to several months following exposure. Mice exposed to asymptomatic doses display long-lasting deficiency in emotional learning due to a region-specific impairment in dopaminergic functions (Lucot JB, personal communication). Interestingly, data on sarin exposures have shown inhibition of new memory formation rather than a degraded memory retrieval process. These observations are consistent with the findings of sarin-induced cognitive and performance deficits in the survivors of the sarin attacks in Japan (Hatta et al., 1996; Yokoyama et al. 1998). Rescue workers and victims, who did not develop any symptoms of acute cholinergic neurotoxicity, also exhibited chronic decline in memory (Nishiwaki et al, 2001; Miyaki et al, 2005).

1.2 Mechanistic Analysis of Sarin-Induced Organ Injury

Given the complexity of cellular response to the toxicity of sarin exposure, a global profiling of the changes in the cellular constituents will be needed in order to elucidate the molecular events following sarin exposure. This approach could significantly facilitate the understanding of the molecular mechanism of sarin-induced organ injury. In addition, it could also facilitate the identification of biomarkers for early detection of the early events of sarin-induced injury. Ultimately, it could guide the development of countermeasures that can effectively treat and/or prevent the onset of organ injury induced by sarin and other organophosphorus (OP) agents.

Of different ~omics technologies, DNA microarray technology is the most powerful profiling technique with the promise of global coverage. Using this technique, previous reports from this laboratory have shown successes in assessing differential gene expression and pathway modulation in response to a toxic insult (Soto et al, 2008; Stapleton et al, 2009; Davidson et al, 2009). Detailed analysis of the patterns of differential gene expression at different time points can reveal temporal progression of cellular events associated with initial defensive response, cellular damage/organ injury, and tissue repair/remodeling. These data, in turn, will provide novel mechanistic insights into the initiation, progression, and persistence of molecular lesions that lead to cellular/tissue pathology and functional deficits in the organs under study.

Although studies addressing different aspects of low-dose sarin (or other OP) treatments have previously been performed using various animal models (Kassa et al, 2001a; Kassa et al, 2001b; Henderson et al, 2002; Hulet et al, 2002; Kassa et al, 2002; Shih et al, 2006; Genovese et al, 2008), studies specifically designed for the investigation of the molecular mechanism(s) of injury in multiple brain regions and multiple vital organs following exposure to sublethal doses of sarin have not been conducted. To address this knowledge gap, a systematic and comprehensive investigation of differential gene expression using DNA microarrays in multiple brain regions and multiple vital organs was proposed, funded by the Defense Threat Reduction Agency (DTRA), and initiated by the AF. A number of brain regions and major peripheral organs from sarin-treated animals were isolated and preserved. These tissues would be invaluable for a comprehensive investigation of sarin-induced differential gene expression and pathway perturbation, which will likely provide novel insights into the underlying mechanisms of sarin-induced organ injury. Integration of pathway perturbation results within these brain regions and peripheral organs will provide an understanding of the molecular mechanism of sarin-induced injury at the organism level. These data will address the initial molecular and mechanistic foundation. This in turn will significantly facilitate future development of pre-exposure and/or post-exposure countermeasures that can prevent and treat overall injury induced by sarin and other OP agents.

1.3 Molecular Mechanism of Sarin-Induced Hippocampal Injury

The brain hippocampus plays an important role in cognitive function(s), including learning and memory (especially spatial memory and the transference of short-term to long-term memory). Hippocampus is also one of the most sensitive brain regions to sarin-induced injury. Because of these reasons, the initial focus of this study is to investigate differential gene expression and pathway perturbation in this brain region. Specifically, differential gene expression in the hippocampal region following sarin exposure was investigated using DNA microarrays in a

mouse model. The DNA microarray data was analyzed to elucidate the molecular mechanism of sarin-induced injury by the analysis of perturbation of biological pathways using advanced bioinformatics. These results as described will provide novel insights into the molecular mechanism(s) of sarin-induced hippocampal injury, as well as the mechanism(s) of sarin-induced cognitive performance degradation as observed in animal studies and in humans (Landauer et al, 1984; Kassa et al, 2001a; Kassa et al, 2001b; Kassa et al, 2001c; Nishiwaki et al, 2001; Hulet et al, 2002; Kassa et al, 2002; Kassa et al, 2004; Miyaki et al, 2005; Genovese et al, 2007; Genovese et al, 2008; Grauer et al, 2008).

2. MATERIALS AND METHODS

Animal exposure was conducted at Wright State University (WSU) under the Henry M. Jackson Foundation Cooperative Agreement FA8650-05-2-6518, Subaward # 667357. All procedures involving live animals were conducted in a facility accredited by the Association for the Assessment and Accreditation of Laboratory Animal Care, International, in accordance with the Guide for the Care and Use of Laboratory Animals, National Research Council (1996). All experiments were performed according to the Animal Use Protocol AUP #810 "*Organ Specific Perturbation of Biological Pathways and Processes Resulted from Exposure to Organophosphorus Agent in Mice (Mus musculus)*". This animal protocol was approved by the WSU Institutional Animal Care and Use Committee (IACUC) on 11August 2009. It was subsequently reviewed and approved by the Wright-Patterson IACUC on September 29, 2009.

2.1 General Procedures

Male Swiss Webster mice (body weight 26–28 g, aged 3 months) (from Harlan Lab.) were kept in a temperature-controlled room (21–23 °C) with a 12-h light/dark and provided with standard diet (Mouse Chow 5015, from Purina, St. Louis, MO) and tap water *ad libitum*. Treatment of animals with sarin was carried out in the Dilute Chemical Surety Facility at Wright State University, which is approved for handling sarin.

2.2 Determination of Mean Lethal Dose (LD50) for Sarin

Since the potency of each batch of sarin received from USAMRICD is different, the LD50 of each of the batches of sarin used in this study were determined experimentally. Groups of 10 animals received a single dose of sarin subcutaneously (*s.c.*) with concentrations starting with the LD50 calculated from historical data and the concentration determined by USAMRICD (i.e. 275-390 µg/kg). Additional concentrations up to plus or minus 10% the LD50 were also used. A 24-hr lethality time point was used to establish the LD50. The surviving animals were sacrificed at the end of the experiment.

2.3 Treatment of Animals with 0.4x, 0.6x and 0.75x LD50 of Sarin

For the studies of sarin-induced differential gene expression and pathway perturbation, three doses (0.4x, 0.6x and 0.75x LD50) of sarin, matched with vehicle controls, were administered via *s.c.* injection. The control and sarin-treated animals were monitored using a functional observational battery (FOB) for signs of treatment-related toxicity, and scores recorded. Since five surviving animals per group are needed for the analysis of differential gene expression, the

group sizes for the doses of 0.6x and 0.75x LD50 were increased to six and eight animals per group, respectively. This is to account for the possibility that some of the animals will be lost prior to the 24 hr experimental endpoint, due to treatment-induced mortality. Sarin-induced differential gene expression at eight time points, 2 hours, 6 hours, 12 hours, 1 day, 2 days, 5 days, 10 days and 25 days post-treatment, was investigated. Table 1 shows the number of animals in each of the sample groups with respect to sarin doses and time points after sarin injection.

Table 1: Number of Animals in Each Treatment Group*

Time Point (hours after sarin injection)	Sarin (GB) Dose (xLD50)			
	0	**0.4**	**0.6**	**0.75**
0	5	0	0	0
2	5	5	6	8
6	5	5	6	8
12	5	5	6	8
24	5	5	6	8
28	5	5	6	8
120	5	5	6	8
240	5	5	6	8
600	5	5	6	8
Total (8 Time points)	45	40	48	64

*Each animal group represents a specific combination of dose and time point.

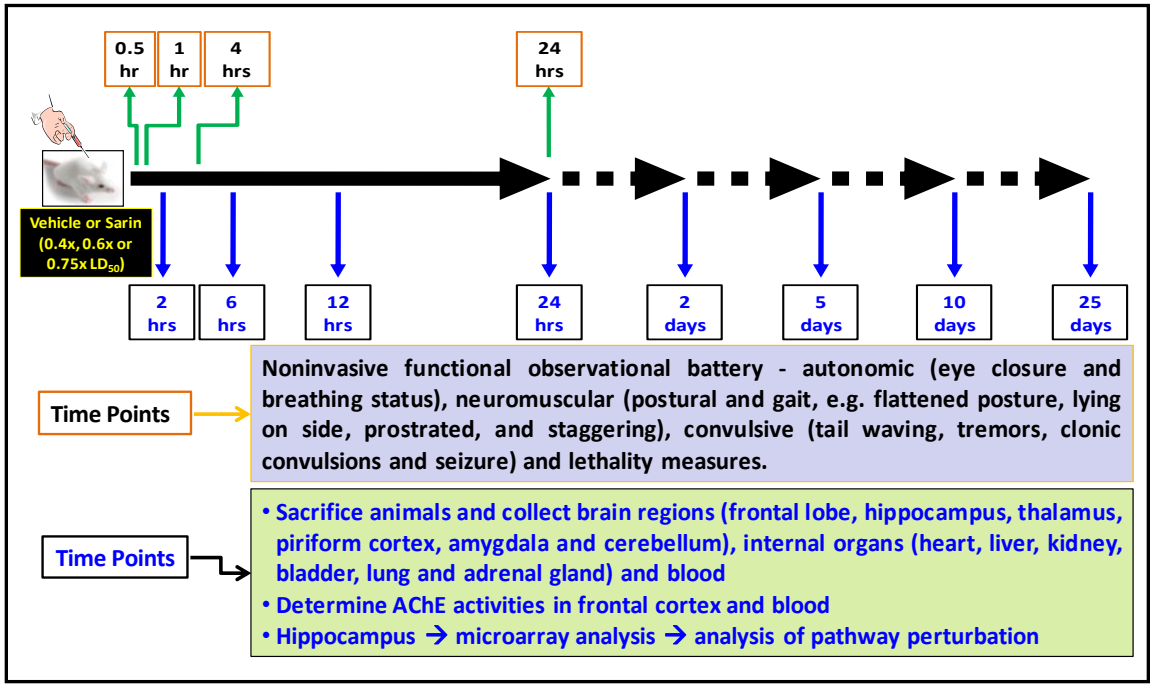


Figure 1: Diagram of Overall Study Design. The time points of FOB evaluation, tissues collection and AChE/BuChE activity assays are illustrated. Brain regions and peripheral organs collected are listed.

Six brain regions (hippocampus, frontal lobe, thalamus, piriform cortex, amygdala and cerebellum) and six peripheral organs (heart, liver, kidney, bladder, lung and adrenal gland) were collected, processed as required and snap-frozen in liquid nitrogen. The frozen tissues were preserved at -80 °C for long-term storage until processing for the analysis of AChE and butyrylcholinesterase (BuChE) inhibition and RNA extraction. The whole blood collected from each animal was split into two halves, one for the isolation of plasma and peripheral blood mononucleated cells for long-term storage at -80 °C, while the other half was used for measuring AChE and BuChE activities. The overall design of this study is shown in Figure 1.

2.4 Evaluation of Sarin-Induced Toxicity

Sarin-induced toxicity was evaluated using two methods:

a. Non-invasive FOB Scoring System; and
b. Inhibition of AChE and BuChE in frontal lobe and plasma/red blood cells.

2.4.1 FOB Assessment

Noninvasive FOB assessment is a checklist of symptoms associated with OP exposure. Signs of behavioral functions of control and sarin-treated animals were directly observed through the housing cage. Handling was eliminated as it is a stressor to the animals that confounds results. The functional categories under observation were autonomic, neuromuscular, and convulsive. Autonomic parameters included eye closure and breathing status. Neuromuscular parameters are primarily postural and gait. These included flattened posture, lying on side, prostrated, and staggering. Convulsive parameters included tail waving, tremors, and convulsions or seizures. The FOB scores were taken at 4 time points (0.5, 1, 4 and 24 hours) after sarin injection. The maximum FOB score is 21 (severely affected by sarin treatment and/or death), while that of an animal showing normal behavioral functions is 6. Additionally, the time of first seizure and the time of death, if occurred, were also recorded. In the case that the death of an animal occurs before any symptoms can be observed, a maximum score of 21 points will be assigned. An example of a FOB Scoring Worksheet is shown in Table 2.

Table 2: FOB Scoring System Worksheet

Behavioral Functions	**Score**			
	1	**2**	**3**	**4**
Posture	normal	flattened	prostration	side / contorted / splayed
Motor Behavior	normal	tail twitching / hypo-loco	tremors	convulsions
Gait Score	normal	staggering / splayed	severe staggering	unable to move
Eyelid	open	half closed	slight protrusion	severe protrusion
Breathing	open	fast shallow	slow labored	
Death	no death	death		
Additional Information				
Body Temp				
Time of first Seizure				
Time of death				
Other Comments				

2.4.2 Inhibition of AChE and BuChE

To evaluate treatment-induced toxicity, AChE and BuChE activities in frontal cortex and blood were analyzed. Frozen brain tissue were homogenized in a buffer (containing 0.1 M sodium phosphate buffer, pH 7.4, and 0.5 % Tween 20) and centrifuged at 13,000 xg for 5 min. Tissue extracts were recovered, and AChE and BuChE activities analyzed according to the Ellman method (Ellman et al., 1961). Additionally, the erythrocyte AChE activity and plasma BuChE activity were determined using the trunk blood collected at the time of sacrifice. Five microliters (µL) of tissue extract or blood was added to the reaction buffer. For each sample, half of each replicate was treated with tetraisopropylpyrophosphoramide (iso-OMPA) to determine the relative activity of AChE (iso-OMPA selectively inhibits BuChE activity). The relative activity of BuChE was determined by subtracting the AChE activity (reaction with iso-OMPA) from the total activity (reaction without iso-OMPA).

The statistical software "R" (version 2.6.2) was used for statistical analysis and graphical presentation of the results of AChE and BuChE activity assays. In general, analysis of variance (ANOVA) was used for data showing a Gaussian distribution. The statistical significance threshold was set at 0.05.

2.5 Collection and Processing Tissue Samples

At eight specific time points (i.e., 2 hours, 6 hours, 12 hours, 1 day, 2 days, 5 days, 10 days and 25 days) after sarin treatment, the control and sarin-treated animals were sacrificed. The brain collected from each animal was washed thoroughly with ice-cold RNase-free water to remove traces of blood, split into two portions, one for AChE activity measurement and the other for gene expression profiling. The half for gene expression was immediately immersed in RNAlater (Ambion, USA) at 4 ˚C to stabilize the transcriptome prior to further dissection to isolate specific brain regions (hippocampus, frontal lobe, thalamus, piriform cortex, amygdala and cerebellum). The dissected brain tissues were preserved at -80 ˚C until processing for total RNA extraction. Other peripheral organs (heart, liver, kidney, bladder, lung and adrenal gland) were also collected, processed and preserved in a similar manner at the time of necropsy. The portion for AChE/BuChE activity measurement was processed immediately to isolate the frontal lobe, which is then snap-frozen in liquid nitrogen and stored at -80˚C.

Blood samples were collected, and split into two equal portions. Erythrocytes, leukocytes and plasma were separated by differential centrifugation using one half of the blood sample. The erythrocytes, leukocytes and plasma were then recovered, snap-frozen and preserved at -80˚C. The other half of the blood sample that is for AChE/BuChE activity assay was snap-frozen in liquid nitrogen and stored at -80˚C.

2.6 Transcriptomic Profiling of Hippocampal Region

Although several brain regions and peripheral organs were collected from the animals, only the hippocampal region was used for transcriptomic profiling in this initial effort. Transcriptomic profiling was performed using the Affymetrix Mouse Exon 1.0 ST GeneChip Arrays. Total RNA was isolated from the hippocampal tissues using Qiagen RNeasy Mini Kit (Qiagen,

Valencia, CA). Frozen tissues were homogenized in lysis buffer containing guanidinium thiocyanate and β-mercaptoethanol. Extraction and purification of total RNA from tissue homogenates were performed according to the manufacturer's instructions. The quality of the isolated RNA was confirmed using the RNA 6000 Nano LabChip Kit with the Agilent 2100 Bioanalyzer System (Agilent Biotechnologies, Palo Alto, CA). RNA concentration was determined using the NanoDrop 1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE).

The purified total RNAs were used for target preparation as described in the user manuals for whole transcript (WT) expression analysis (Ambion WT Expression Kit and Affymetrix GeneChip WT Expression Array Kit). Briefly, 50 ng total RNA was used for first-strand cDNA synthesis in the presence of oligo (dT)-T7 promoter primers. The resulting first-strand cDNA was then converted to double-stranded cDNA, which was then used as template for *in vitro* transcription. The synthesized cRNA was purified by binding to magnetic Nucleic Acid Binding Beads, followed by several washes in isopropanol and Nucleic Acid Wash Solution. The cRNA was then eluted using elution solution preheated to 56 ˚C. The purified cRNA was then used for the second round of cDNA synthesis in the presence of random primers. After the cDNA synthesis was completed, the cRNA template was removed by RNase H digestion. The resulting single-stranded cDNA was purified using the magnetic Nucleic Acid Binding Beads as described above. The purified single-stranded cDNA was fragmented to reduce its size. The fragmented cDNA was purified using the Affymetrix Sample Clean-Up Module Kit. The purified single-stranded cDNA was then labeled with biotin using Terminal deoxynucleotidyl transferase. The biotin-labeled cDNA was purified using the Affymetrix Clean-Up Module Kit. The concentration and purity of each labeled cDNA sample were determined by measuring A_{260} and the ratio of A_{260}/A_{280}, respectively.

Hybridization cocktail, which contained (for each sample) 5.5 µg of labeled cDNA, Affymetrix control oligo B2, Affymetrix eukaryotic hybridization control, herring sperm DNA, acetylated BSA and dimethyl sulfoxide in 1x hybridization buffer (final concentration) was heated to 99 ˚C for 5 min, then cooled to 45 °C for 5 min before being loaded onto an Affymetrix Mouse Exon 1.0 ST GeneChip array. Hybridization was performed at 45 ˚C for 16 hours in an Affymetrix hybridization oven with constant end-over-end rotation at 60 RPM. Once hybridization was complete, hybridization mix was removed from each GeneChip array, and the microarrays washed and stained in the Affymetrix Gene-Chip Fluidics Station 450 using the EukGE_Ws2v5 protocol. After washing and staining, the microarrays were scanned in the Affymetrix GeneChip Scanner 3000 using the Affymetrix GeneChip Command Console Software (AGCC) software. The scanned images were stored as .dat files. Data obtained from the Affymetrix GeneChip Scanner 3000 were then processed using the Affymetrix Expression Console Software for quality control analysis. Only the microarrays that passed the quality control test were included in the down-stream data analysis.

2.7 Identification of Sarin-Induced Hippocampal Gene Expression Changes and Pathway Perturbation

To identify sarin-induced hippocampal gene expression changes, the data files were exported from the Affymetrix Expression Console and imported into the Biotique XRAY software.

Statistical analysis of significant gene expression changes was performed using sarin treatment as the study factor with the significance threshold set at 0.05. The option of all pair-wise comparison was selected to identify significant gene expression changes between the control and all dose groups, as well as that among all dose groups. Multiple test correction was implemented using the Holm's Step-down Bonferroni Method (Holm, 1979). The "extended probe sets" filter, which includes transcript clusters supported by RefSeq, Ensembl Transcripts, full-length mRNAs, cDNA-based annotations and other genomic annotations of medium reliability, was used. This filter provided a total of ~129,000 transcripts (including alternatively spliced products). To ensure data quality, probe sets with a GC count <6 or >17, as well as that with <3 probes per probe set, were excluded from the analysis. Non-expressed probe sets (signals <10% above background or detection significance threshold >0.05) were also excluded. Finally, a fold change threshold >1.2 was implemented.

To group the differentially expressed genes with a similar dose- or temporal response pattern in terms of expression changes, the differentially expressed genes were subjected to self-organizing map (SOM) clustering analysis. In addition, the lists differentially expressed genes were exported as text files for pathway analysis and biological interpretation. Finally, to identify pathways with statistically significant gene enrichment ($p<0.05$), the lists of up- and down-regulated genes, as well as the list of all differentially expressed genes, were used as input files to search the biological annotation databases (e.g. the Gene Ontology and the KEGG pathway databases) in the Database for Annotation, Visualization, and Integrated Discovery (DAVID) website (Huang et al, 2007).

3. RESULTS

3.1 Sarin-Induced Toxicity

3.1.1 FOB Assessment

In this study, mice were treated by subcutaneous injection with sublethal doses of sarin ranging from 0.4x – 0.75x LD50. For logistic reasons, each time point was conducted as an independent experiment (see Table 1 for details). Of all sarin-treated animals, only 1 animal died. However, 38 animals (~60%) receiving sarin treatment at the dose of 0.75x LD50 had seizure, with >80% of these events occurring within 20 minutes after sarin injection. Table 3 shows the time of first seizure occurrence in the animals that were positive for sarin-induced seizure.

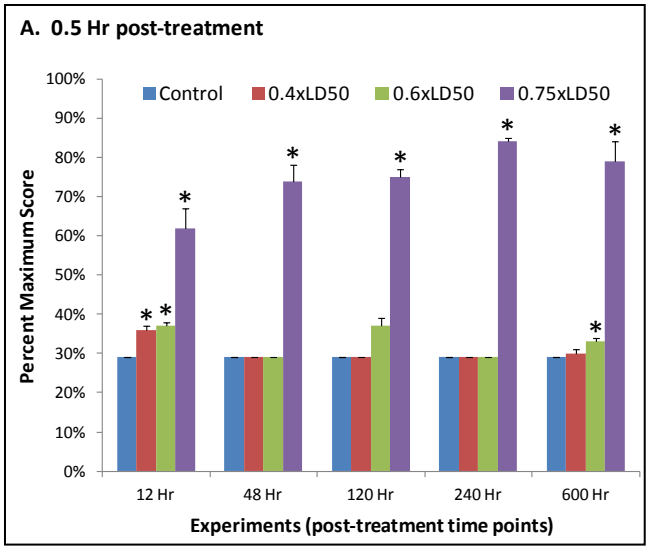
Other symptoms associated with OP exposure were also evaluated using the FOB scoring system. The functional categories under observation were autonomic parameters (including eye closure and breathing status), neuromuscular parameters (primarily postural and gait including flattened posture, lying on side, prostrated, and staggering), convulsive parameters (including tail waving, tremors, convulsions and seizures). As shown in Figure 2 (see Supplemental Table 1 for detailed scores), animals with sarin treatment at 0.4x and 0.6x LD50 were mostly asymptomatic or minimally symptomatic, while animals treated with 0.75x LD50 showed symptoms characteristic of OP exposure with statistically significant increases in FOB scores, especially at early time points up to 4 hours post-treatment. Most of these symptoms emerged within 30 minutes after sarin treatment and maintained at the peak level within the first hour. However, the

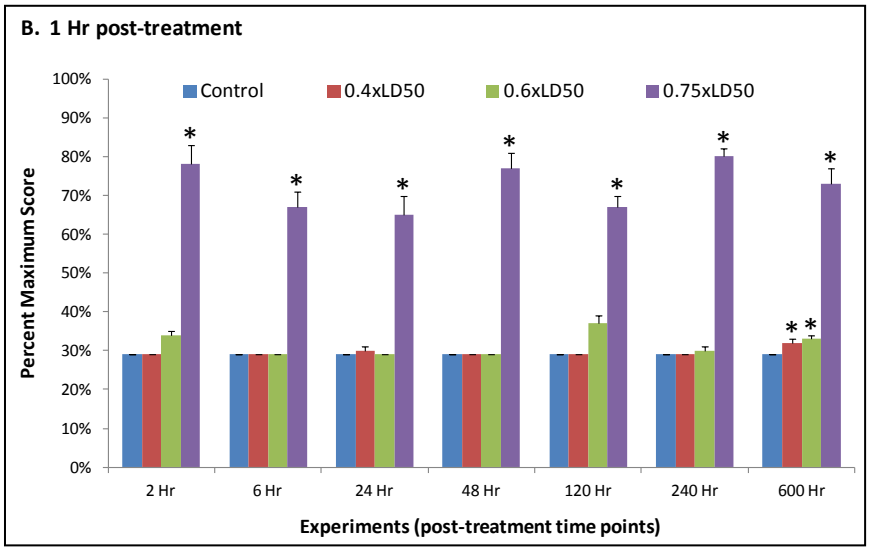
animals showed some degree of recovery at 4 hours post-treatment. By 24 hours, almost all animals showed complete recovery from sarin-induced toxicity, with respect to FOB scores.

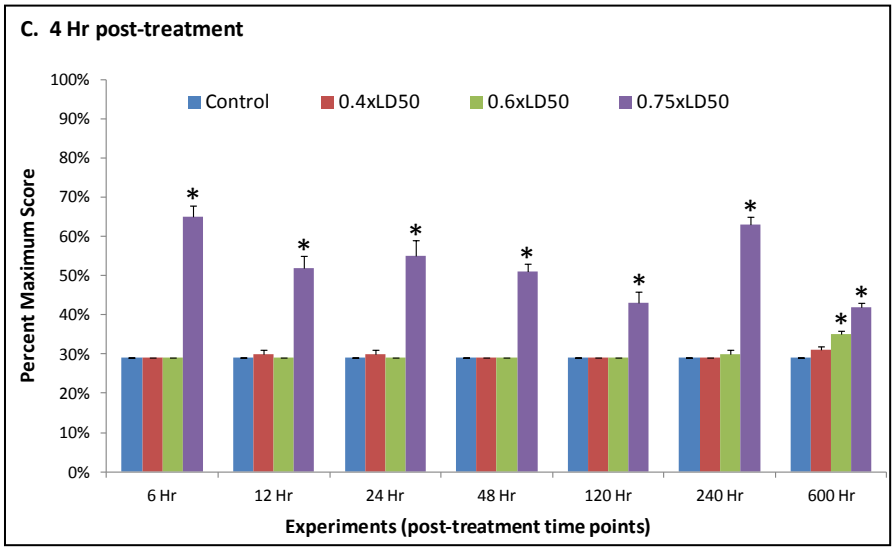
Table 3: Percentage of Animals with Seizure and Time of First Seizure Occurrence in the 0.75x LD50 Dose Group at Each Time Point.

Sacrifice Time Point (post-injection)	Animal	Time of first seizure (m:s)	% with Seizure
2 Hr	1	6:01	**62.5%**
	2*	7:07	
	3	13:05	
	4	16:16	
	5	17:16	
6 Hr	1	11:27	**37.5%**
	2	16:37	
	3	20:03	
12 Hr	1	1:29	**37.5%**
	2	1:33	
	3	1:40	
24 Hr	1	9:50	**50.0%**
	2	10:57	
	3	12:54	
	4	19:09	
2 Days	1	10:06	**75.0%**
	2	11:39	
	3	14:50	
	4	19:25	
	5	26:52	
	6	52:42	
5 Days	1	11:03	**50.0%**
	2	11:05	
	3	11:08	
	4	25:30	
10 Days	1	8:04	**100.0%**
	2	9:11	
	3	10:17	
	4	13:00	
	5	13:03	
	6	14:26	
	7	14:38	
	8	17:10	
25 Days	1	10:51	**62.5%**
	2	11:10	
	3	16:07	
	4	16:15	
	5	38:42	

*Treatment-induced mortality was observed.







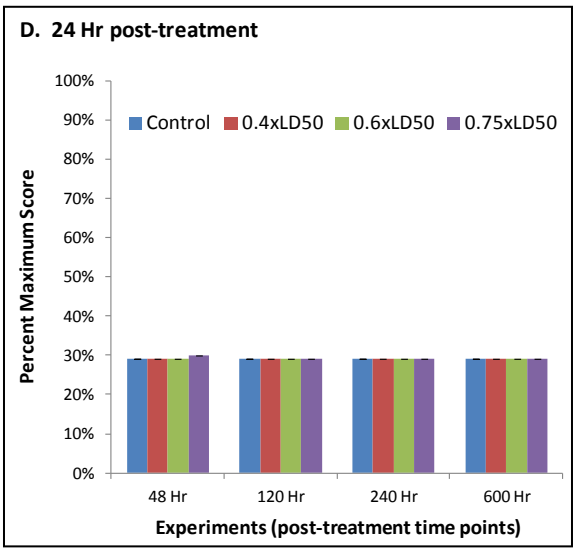


Figure 2: FOB Scores of Control and Sarin-treated Animals. The control and treated animals were monitored at 4 time points after sarin treatment and scored according to the FOB Scoring System as described above. (A) Animals were observed at 30 minutes post-treatment; data of three experiments, 2-hr, 6-hr and 24-hr time points, is not available. (B) Animals were observed at 1 hour post-treatment; data of the 12-hr time point experiment is not available. (C) Animals were observed at 4 hour post-treatment; animals of the 2-hr time point experiment have been sacrificed. (D) Animals were observed at 24 hour post-treatment; animals of the experiments for the first four time points (i.e., 2, 6, 12 and 24 hours) have been sacrificed. X-axis = time points at which animals were sacrificed. Y-axis = average percentage of maximum score (21 points). Error bar = standard error. ***** = Significant difference (p<0.05).

3.2 Sarin-Induced Toxicity - Inhibition of AChE and BuChE

Sarin-induced toxicity was also assessed by inhibition of AChE and BuChE in the frontal lobe. Consistent with the FOB results, sarin treatment at 0.4x LD50 did not resulted in significant changes in AChE activity, except that a few cases showed statistically significant (but small) inhibition of AChE (Figure 3). Although sarin treatment at 0.6x LD50 resulted in minimal changes in behavioral functions, significant inhibition (i.e. ~50%) of AChE (and BuChE) was observed, especially within the first 6 hours after treatment. Significant inhibition of AChE, at ~35%, persisted up to 10 days post-treatment. Even at 25 days after sarin treatment, ~20% inhibition of AChE could still be observed. However, the changes at the late time points did not reach statistical significance. As expected, sarin treatment at 0.75x LD50 resulted in dramatic inhibition (>60%) of AChE (and BuChE) in the frontal lobe (for details, see Supplemental Table 2).

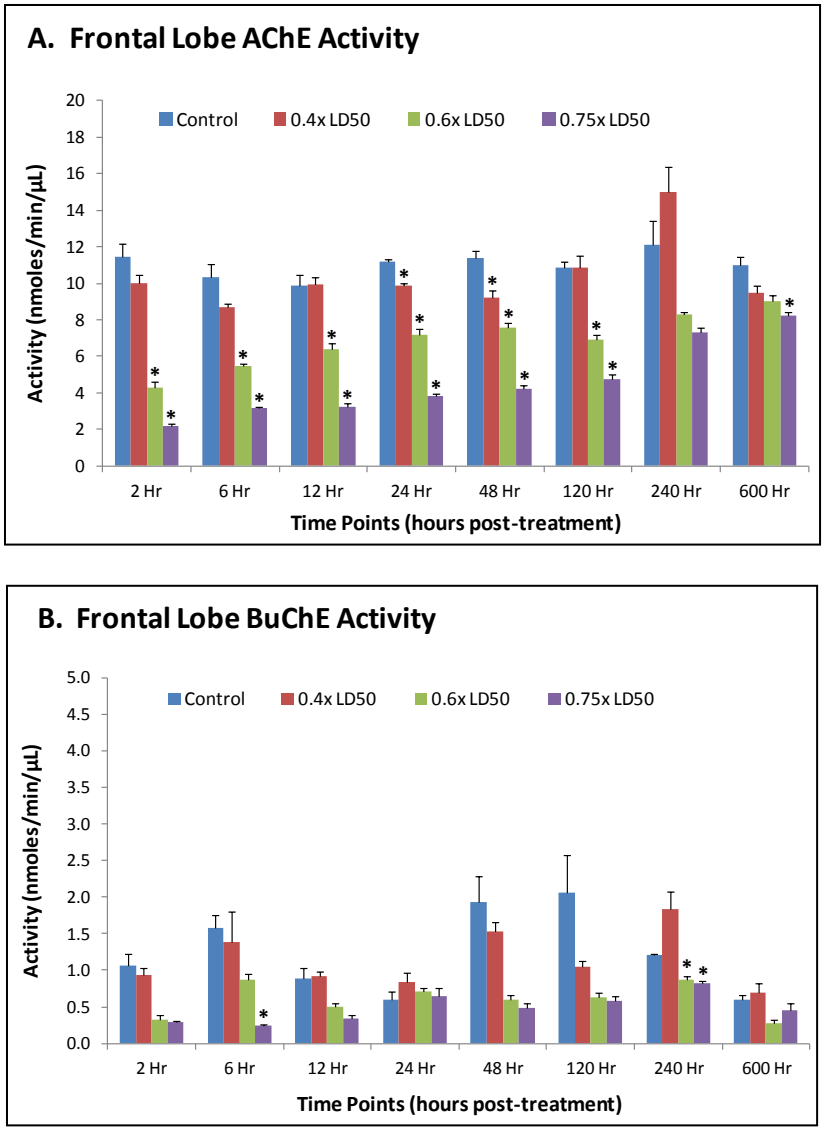


Figure 3: Frontal Lobe AChE and BuChE Activities. AChE activity was determined using the Ellman Method (Ellman et al., 1961). Statistically significant inhibition of AChE in the 0.6x and 0.75x LD50 dose groups was observed up to 5 days post-treatment. (A) AChE activity. (B) BuChE activity. X-axis = time points after sarin treatment. Y-axis = Enzyme activity (nmoles/min/µL). Error bar = standard error. * = Significant inhibition (p<0.05) of AChE and BuChE.

Inhibition of AChE and BuChE in the blood was also determined. In contrast to the FOB and frontal lobe AChE results, sarin treatment at 0.4x LD50 resulted in significant inhibition (≥50%) of erythrocyte AChE up to 12 hours (Figure 4). However, the enzyme activity recovered at later time points. Similarly, sarin treatment resulted in significant inhibition of plasma BuChE in all doses at 2 hours post treatment, which is followed by a rapid recovery of the enzyme activity. Blood AChE and BuChE activities in control and sarin-treated animals are shown in Supplemental Table 3.

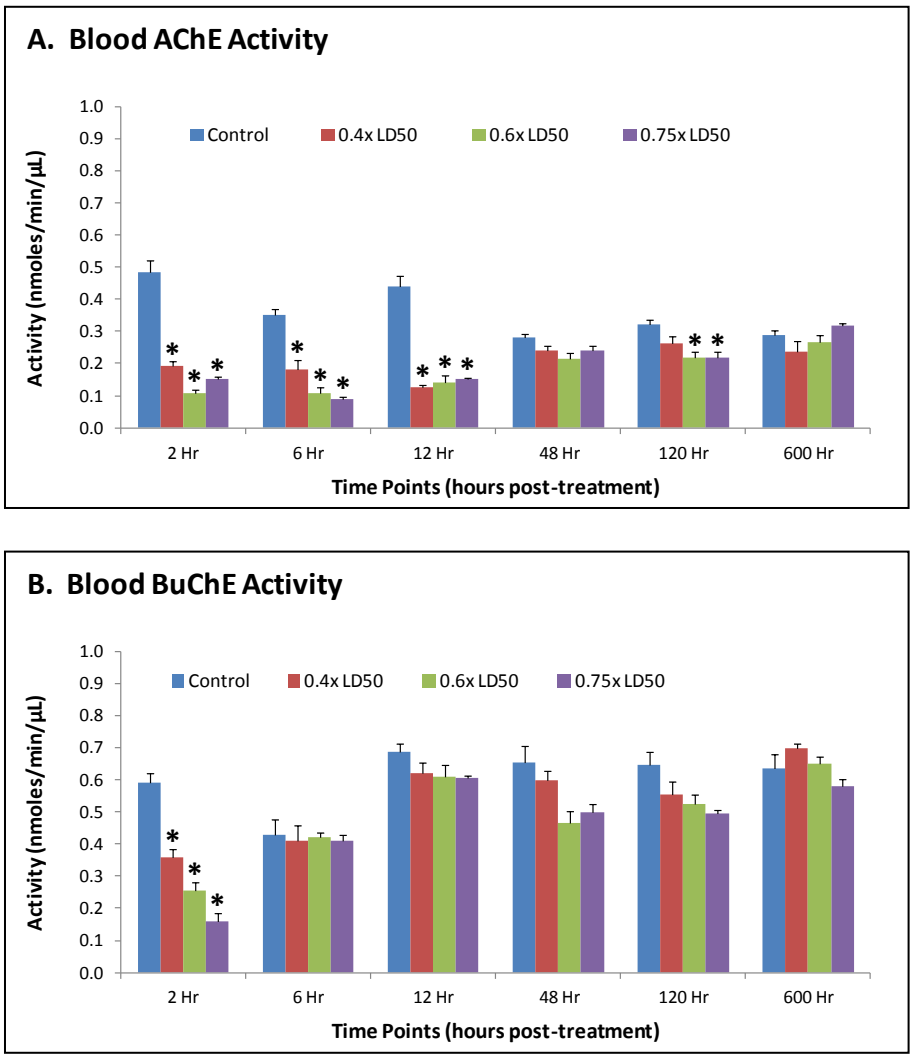


Figure 4: Blood AChE and BuChE Activities. AChE activity was determined using the Ellman Method (Ellman et al., 1961). Statistically significant inhibition of erythrocyte AChE in the sarin treatment groups was observed up to 12 hours post-treatment. (A) Erythrocyte AChE activity. (B) Plasma BuChE activity. X-axis = time points after sarin treatment. Y-axis = Enzyme activity (nmoles/min/µL). Error bar = standard error. * = Significant inhibition (p<0.05) of AChE and BuChE.

3.3 Sarin-Induced Hippocampal Gene Expression Changes

Statistical analysis of the microarray data revealed that sarin treatment resulted in differential expression of a large number of genes (Figure 5 and Supplemental Table 4).

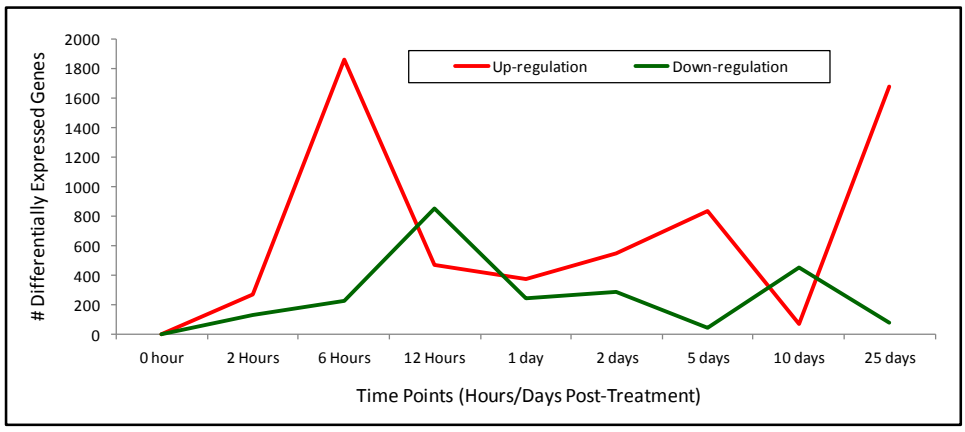


Figure 5: Sarin-Induced Hippocampal Differential Gene Expression. The number of up- and down-regulated genes in the hippocampal region following sarin treatment is shown.

3.3.1 Dose Response of Hippocampal Gene Expression Following Sarin Treatment

The dose response of the differentially expressed genes at each time points is determined using the SOM clustering analysis, an unsupervised pattern recognition technique, with a 3x3 setting to generate nine clusters. The results are summarized in Tables 4, A-H (the SOMs at these time points are shown in Supplemental Figure 1, A-H).

Table 4: Dose Response of Sarin-Induced Hippocampal Differential Gene Expression. The result of SOM clustering analysis revealed that the differentially expressed genes showed unique dose response patterns after sarin treatment. The result at each time point is shown separately.

A. 2 Hours Post-treatment									
Cluster #	**0.4xLD50**	**0.6xLD50**	**0.75xLD50**	**# Genes**		**Dose Response**	**Total**	**Trend**	**Pattern**
Cluster 1	Up	Same as 0.4xLD50	Up more	150	163	0.4=0.6 < 0.75	163	Up	Highest: 0.75xLD50
Cluster 4	Up	Same as 0.4xLD50	Up more	13					
Cluster 2	Up	Less than 0.4xLD50	Less than 0.4xLD50	49	49	0.4 > 0.6=0.75	80	Up	Highest: 0.4xLD50
Cluster 3	Up	No change	No change	31	31	0.4 > 0.6=0.75			
Cluster 6	Up	Down	Down more	17	17	0.4 > 0.6=0.75	17	Variable	Highest: 0.4xLD50
Cluster 7	No change	Up	Up more	31	31	0.4 < 0.6 < 0.75	31	Up	Progressively up
Cluster 8	Down	No change	No change	4	4	0.4 < 0.6=0.75	4	Down	0.4xLD50 only
Cluster 9	Down	Same as 0.4xLD50	Same as 0.4xLD50	109	109	0.4 = 0.6 = 0.75	109	Down	Dose independent
Cluster 5	N/A	N/A	N/A	0	0	N/A	0	N/A	N/A

B. 6 Hours Post-treatment									
Cluster #	**0.4xLD50**	**0.6xLD50**	**0.75xLD50**	**# Genes**		**Dose Response**	**Total**	**Trend**	**Pattern**
Cluster 1	Up	Same as 0.4xLD50	Up more	124	156	0.4=0.6 < 0.75	156	Up	Highest: 0.75xLD50
Cluster 5	Up	Same as 0.4xLD50	Up more	32					

Cluster 8	Up	More than 0.4xLD50	Same as 0.4xLD50	202	438	0.4=0.75 < 0.6	438	Up	Bell-shaped
Cluster 9	Up	More than 0.4xLD50	Same as 0.4xLD50	236					
Cluster 2	Up	Less than 0.4xLD50	Same as 0.4xLD50	251	1050	0.4=0.75 > 0.6	1050	Up	U-shaped
Cluster 3	Up	Less than 0.4xLD50	Same as 0.4xLD50	370					
Cluster 6	Up	Less than 0.4xLD50	Same as 0.4xLD50	429					
Cluster 4	Up	Less than 0.4xLD50	Same as 0.6xLD50	7	7	0.4 > 0.6=0.75	7	Up	Highest: 0.4xLD50
Cluster 7	No change	Up	Down	437	437	0.6 < 0.4 < 0.75	437	Variable	Up then down

C. 12 Hours Post-treatment

Cluster #	0.4xLD50	0.6xLD50	0.75xLD50	# Genes		Dose Response	Total	Trend	Pattern
Cluster 6	Up	Same as 0.4xLD50	Up more	423	423	0.4=0.6 < 0.75	423	Up	Highest: 0.75xLD50
Cluster 5	Up	No change	No change	1	1	0.4 > 0.6=0.75	1	Up	0.4xLD50 only
Cluster 7	Down	Down more	Same as 0.6xLD50	529	529	0.4 > 0.6=0.75	529	Down	Highest: 0.4xLD50
Cluster 3	Up	Down	Up	37	37	0.4=0.75 > 0.6	112	Variable	U-shaped
Cluster 2	No change	Down	No change	75	75	0.4=0.75 > 0.6		Down	U-shaped
Cluster 9	No change	Up	No change	9	9	0.4=0.75 < 0.6	9	Up	Bell-shaped
Cluster 8	No change	No change	Down	28	28	0.4=0.6 > 0.75	28	Down	0.75xLD50 only
Cluster 1	No change	Down	Down, >0.6xLD50	216	220	0.4 > 0.75 > 0.6	220	Down	0.4xLD50 - no effect
Cluster 4	No change	Down	Down, >0.6xLD50	4					

D. 24 Hours Post-treatment

Cluster #	0.4xLD50	0.6xLD50	0.75xLD50	# Genes		Dose Response	Total	Trend	Pattern
Cluster 6	Up	Same as 0.4xLD50	Up more	35	35	0.4=0.6 < 0.75	35	Up	Highest: 0.75xLD50
Cluster 5	No change	No change	Up	12	67	0.4=0.6 < 0.75	67	Up	0.75xLD50 only
Cluster 3	No change	No change	Up	28				Up	
Cluster 8	No change	No change	Up	27				Up	
Cluster 2	Down	Same as 0.4xLD50	Up	25	25	0.4=0.6 < 0.75	25	Variable	Down then up
Cluster 1	Down	Same as 0.4xLD50	No change	231	231	0.4=0.6 < 0.75	231	Down	0.75xLD50 – no effect
Cluster 9	Up	Same as 0.4xLD50	Same as 0.4xLD50	256	256	0.4 = 0.6 = 0.75	256	Up	Dose independent
Cluster 7	No change	Down	Up	2	2	0.6 < 0.4 < 0.75	2	Variable	Down then up
Cluster 4	No change	Down	No change	4	4	0.4=0.75 > 0.6	4	Down	U-shaped

E. 2 Days Post-treatment

Cluster #	0.4xLD50	0.6xLD50	0.75xLD50	# Genes		Dose Response	Total	Trend	Pattern
Cluster 8	Up	Up more	Same as 0.4xLD50	45	45	0.4=0.75 < 0.6	45	Up	Bell-shaped
Cluster 9	Up	Same as 0.4xLD50	Same as 0.4xLD50	258	258	0.4 = 0.6 = 0.75	258	Up	Dose independent

Cluster 7	Up (x2)	Same as 0.4xLD50	Down	7	7	0.4=0.75 > 0.6	7	Variable	Up then down
Cluster 5	Up	No change	Same as 0.4xLD50	3	167	0.4=0.75 > 0.6	167	Up	U-shaped
Cluster 6	Up	No change	Same as 0.4xLD50	164					
Cluster 4	Up	No change	Down	1	1	0.4 > 0.75 > 0.6	1	Variable	Up then progressively down
Cluster 2	No change	Down	Up	18	91	0.6 < 0.4 < 0.75	91	Variable	Down then up
Cluster 3	No change	Down	Up	73					
Cluster 1	Down	Down more	Down more	275	275	0.4 > 0.6 > 0.75	275	Down	Progressively down

F. 5 Days Post-treatment									
Cluster #	**0.4xLD50**	**0.6xLD50**	**0.75xLD50**	**# Genes**		**Dose Response**	**Total**	**Trend**	**Expression Pattern**
Cluster 1	Up	Up more	Up more	137	229	0.4 < 0.6 < 0.75	229	Up	Progressively up
Cluster 2	Up	Up more	Up more	92					
Cluster 3	Up	Same as 0.4xLD50	Up more	80	80	0.4=0.6 < 0.75	80	Up	Highest: 0.75xLD50
Cluster 4	Up	Same as 0.4xLD50	Same as 0.4xLD50	22	266	0.4 = 0.6 = 0.75	266	Up	Dose independent
Cluster 5	Up	Same as 0.4xLD50	Same as 0.4xLD50	24					
Cluster 7	Up	Same as 0.4xLD50	Same as 0.4xLD50	220					
Cluster 9	Up	Up. <0.4xLD50	Same as 0.4xLD50	100	100	0.4=0.75 > 0.6	100	Up	U-shaped
Cluster 8	Up	Up, <0.4xLD50	Same as 0.6xLD50	99	99	0.4 > 0.6=0.75	99	Up	Highest: 0.4xLD40
Cluster 6	Up	Down	No change	109	109	0.4 > 0.75 > 0.6	109	Variable	Up then down

G. 10 Days Post-treatment									
Cluster #	**0.4xLD50**	**0.6xLD50**	**0.75xLD50**	**# Genes**		**Dose Response**	**Total**	**Trend**	**Pattern**
Cluster 9	No change	No change	Down	28	28	0.4=0.6 > 0.75	186	Down	0.75xLD50 only
Cluster 7	Down	Same as 0.4xLD50	Down more	158	158	0.4=0.6 > 0.75		Down	Lowest: 0.75xLD50
Cluster 6	No change	Up	No change	89	89	0.4=0.75 < 0.6	89	Up	Bell-shaped
Cluster 1	Down	Down, >0.4xLD50	Same as 0.4xLD50	106	106	0.4=0.75 < 0.6	106	Down	Bell-shaped
Cluster 2	Down	No change	Same as 0.4xLD50	43	65	0.4=0.75 < 0.6	65	Down	Bell-shaped
Cluster 3	Down	No change	Same as 0.4xLD50	15					
Cluster 5	Down	No change	Down more than 0.4xLD50	7		0.6 > 0.4 > 0.75		Down	
Cluster 8	Down	Same as 0.4xLD50	Down more	71	71	0.4=0.6 > 0.75	71	Down	Lowest: 0.75xLD50
Cluster 4	Down	Down, >0.4xLD50	Down, >0.4xLD50	13	13	0.6 > 0.4 > 0.75	13	Down	Bell-shaped

H. 25 Days Post-treatment									
Cluster #	**0.4xLD50**	**0.6xLD50**	**0.75xLD50**	**# Genes**		**Dose Response**	**Total**	**Trend**	**Pattern**
Cluster 3	Up	Up more	Up more	132	132	0.4 < 0.6 < 0.75	388	Up	Progressively up

Cluster 6	Down	Up	Up more	256	256	0.4 < 0.6 < 0.75		Variable	Down then progressively up
Cluster 2	Up	Up more	Same as 0.6xLD50	208	888	0.4 < 0.6=0.75	888	Up	Highest: 0.75xLD50
Cluster 4	Up	Up more	Same as 0.6xLD50	47					
Cluster 5	Up	Up more	Same as 0.6xLD50	64					
Cluster 7	Up	Up more	Same as 0.6xLD50	407					
Cluster 8	Up	Up more	Same as 0.6xLD50	162					
Cluster 9	No change	Up	Same as 0.6xLD50	196	196	0.4 < 0.6=0.75	196	Up	0.4xLD50 – no effect
Cluster 1	Up	Same as 0.4xLD50	Same as 0.4xLD50	290	290	0.4 = 0.6 = 0.75	290	Up	Dose independent

Despite the seemingly complex patterns of the clustering results, the dose response can generally be grouped in three categories:

1. Dose-dependent genes: These genes showed progressively up- or down-regulation along the dose range. In some cases, treatments with lower doses did not result in gene expression changes. This pattern of gene expression could be related to defense/repair response. Alternatively, they could also be related to toxicity.

2. Dose independent genes: These genes showed up- or down-regulation at the lowest dose. Treatments with higher doses did not result in larger changes in expression level. This pattern of gene expression is likely related to toxic response. However, their roles in defense/repair could not be ruled out without further verification.

3. Genes with altered response: These genes showed a response pattern with transitional changes. For instance, they were up-regulated at the lowest dose. However as the dose increases, they became down-regulated. An inverse pattern of such response was also observed. These genes are probably involved in the defense/repair response at the low dose and treatment-induced toxicity at the high dose.

Table 5 summarized the percentage of each category of the dose response at all eight time points. Dose dependent genes represent the majority (48-74%) of differentially expressed genes at three time points, 2 hours, 12 hours and 25 days post-treatment, while the genes showing altered response/transitional changes dominated the gene expression changes at the 6-hour time point. Interestingly, the differentially expressed genes almost evenly distribute across the three categories at two time points, 2 days and 5 days post-treatment. At two time points, the differentially expressed genes almost evenly distributed across two of the three categories, dose-independent and altered response categories at 24 hours, and dose-dependent and altered response categories at 10 days post-treatment.

Table 5: Distribution of Differentially Expressed Genes across Dose-Dependent, Dose-Independent and Altered Response Categories

Time Points	Dose Response (% differentially Expressed Genes)		
	Dose-Dependent	Dose-Independent	Variable
2 Hours	48%	27%	25%
6 Hours	7%	0%	93%
12 Hours	74%	0%	26%
24 Hours	16%	41%	42%
2 Days	33%	31%	37%
5 Days	35%	30%	35%
10 Days	51%	0%	49%
25 Days	69%	16%	15%

3.3.2 Temporal Response of Hippocampal Gene Expression Following Sarin Treatment

Detailed examination of the lists of differentially expressed genes at the early and late time points allowed the identification of early-, delayed- and late-response genes, as well as the genes that showed persistent expression changes. Selected genes of interest are listed in the Tables 9 – 12.

Table 6: Selected Early-Response Genes Following Sarin Treatment.

Gene Symbol*	Time Point (Hr)	Expression Level (Control)**	Post-Treatment Fold Change***			
			Control	0.4xLD50	0.6xLD50	0.75xLD50
Ddit4	2	384.68	1.00	1.10	1.25	1.48
	24		1.00	1.00	-1.04	-1.20
Dusp1	2	547.52	1.00	-1.07	1.03	2.15
	48		1.00	-1.01	-1.11	-1.39
Npas4	2	261.65	1.00	1.25	1.16	4.26
	6		1.00	1.70	1.93	2.43
	12		1.00	1.54	1.51	1.88
	120		1.00	-1.16	-1.61	-1.98
Nptx2	6	250.11	1.00	-1.04	1.06	1.34
	12		1.00	-1.15	1.09	1.24
	24		1.00	1.07	1.11	1.91
	48		1.00	1.07	1.07	1.35
Prps2	6	920.06	1.00	-1.19	-1.09	-1.38
	48		1.00	1.01	-1.17	-1.29
Sept11	2	227.14	1.00	-1.56	-1.27	-1.47
	12		1.00	-1.46	-1.42	-1.48
Slc22a8	6	577.19	1.00	1.03	1.06	-1.37
	24		1.00	-1.33	-1.22	-1.22
	48		1.00	-1.07	-1.09	-1.32

*Genes showed significant expression changes within 6 hours after sarin treatment.
**Average value of the relative expression levels of controls at the corresponding time points.
***Negative fold change indicates down-regulation.

A brief description of the presently known functions of these early-response genes is shown below:

a. **Ddit4** (**DNA damage-inducible transcript 4 protein)**: Ddit4 is downstream of AKT1. It inhibits cell growth by regulating the TSC1-TSC2 complex in the TOR signaling pathway. It can promote neuronal cell death. It is up-regulated in substantia nigra neurons from Parkinson disease patients. Ddit4 can be induced by DNA alkylation, ionizing radiation, amyloid beta-peptide and hypoxia.
b. **Dusp1 (Dual specificity phosphatase 1)**: Dusp1 plays an important role in the cellular response to environmental stress. It negatively regulates cell proliferation. Dusp1inactivates mitogen-activated protein (MAP) kinase by concomitant dephosphorylation of both its phosphothreonine and phosphotyrosine residues. Dusp1 can be induced by oxidative stress, heat shock and growth factors.
c. **Npas4 (Neuronal PAS domain protein 4)**: It functions as a transcriptional activator in the presence of ARNT (aryl hydrocarbon receptor nuclear translocator). Npas4 activates the expression of the drebrin gene, which encodes for a cytoplasmic actin-binding protein that has a role in the process of neuronal growth, memory formation and Alzheimer's disease.
d. **Nptx2 (Neuronal pentraxin II)**: Nptx2 is a synaptic protein that is related to C-reactive protein. It is involved in excitatory synapse formation. It also has a role in the clustering of alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) glutamate receptors at established synapses, resulting in non-apoptotic cell death of dopaminergic nerve cells. Up-regulation of Nptx2 in Parkinson's disease (PD) tissues suggests that the protein may be involved in the pathology of PD.
e. **Prps2 (phosphoribosyl pyrophosphate synthetase 2)**: Prps2 encodes a phosphoribosyl pyrophosphate synthetase that plays a central role in the synthesis of purines and pyrimidines.
f. **Sept11 (Septin 11)**: Sept11 belongs to the septin family of filament-forming cytoskeletal GTPases that are involved in cytokinesis and vesicle trafficking. Sept11 may play a role in the neuronal cytoarchitecture, dendritic arborization and dendritic spines, and synaptic connectivity in GABAergic neurons.
g. **Slc22a8 (Solute carrier family 22 (organic anion transporter), member 8)**: Slc22a8 is involved in the sodium-independent transport and excretion of organic anions, some of which are potentially toxic. It has an important role in the excretion/detoxification of endogenous and exogenous organic anions, especially from the brain and kidney.

Table 7: Selected Delayed-Response Genes Following Sarin Treatment.

Gene Symbol*	Time Point (Hr)	Expression Level (Control)**	Post-Treatment Fold Change***			
			Control	0.4xLD50	0.6xLD50	0.75xLD50
Acvr1c	12	265.99	1.00	1.01	-1.19	1.07
	24		1.00	-1.08	-1.45	1.30
	48		1.00	1.10	1.02	1.52
	240		1.00	1.01	1.43	1.37
Ccnt1	12	889.74	1.00	-1.17	-1.48	-1.45
	240		1.00	-1.12	-1.23	-1.30
Hectd2	12	141.62	1.00	1.06	1.05	1.39
	24		1.00	1.15	1.08	1.30
	48		1.00	1.09	1.11	1.38
	600		1.00	1.01	1.18	1.21
Igfbp5	12	1057.11	1.00	-1.00	-1.23	-1.28
	24		1.00	-1.29	-1.15	-1.33
	48		1.00	-1.07	-1.14	-1.42
Nat13	12	497.75	1.00	1.27	1.36	1.41
	48		1.00	1.04	-1.04	1.45
Nd6	12	686.11	1.00	-1.27	-1.25	-1.49
	120		1.00	1.49	1.47	1.57
	240		1.00	-1.14	-1.29	1.99
Nrn1	24	1492.70	1.00	1.01	1.25	1.39
	48		1.00	1.08	1.17	1.36
Ptgs2	24	397.31	1.00	1.15	1.24	1.81
	48		1.00	1.05	-1.11	1.41
	120		1.00	-1.05	-1.21	1.09
Scube1	24	302.68	1.00	1.07	1.02	1.46
	48		1.00	1.01	-1.02	1.35

*Genes showed significant expression changes at or after 12 hours following sarin treatment.
**Average value of the relative expression levels of controls at the corresponding time points.
***Negative fold change indicates down-regulation.

A brief description of the presently known functions of these delayed-response genes is shown below:

a. **Acvr1c (Activin A receptor, type IC)**: Acvr1c is a type I receptor for the TGFβ family of signaling molecules. Acvr1c forms a receptor complex upon ligand binding. Acvr1c has serine/threonine protein kinase activity. Acvr1c plays a role in cell differentiation, growth arrest and apoptosis.
b. **Ccnt1 (Cyclin T1)**: Ccnt1 belongs to the highly conserved cyclin family. It tightly associates with CDK9 kinase, forming a complex called positive transcription elongation factor B (P-TEFb), which can facilitate the transition from abortive to productive elongation by phosphorylating the carboxyl-terminal domain of the large subunit of RNA polymerase II
c. **Hectd2 (HECT domain containing 2)**: Hectd2 functions as an E3 ubiquitin-protein ligase that accepts ubiquitin from an E2 ubiquitin-conjugating enzyme and directly transfers the ubiquitin to targeted substrates.

d. **Igfbp5 (Insulin-like growth factor binding protein 5)**: Igfbp5 prolongs the half-life of the IGFs and can either inhibit or stimulate the growth promoting effects of IGFs on cell culture. It can also alter the interaction of IGFs with their cell surface receptors.
e. **Nat13 (N-acetyltransferase 13)**: Nat13 catalyzes the transfer of acetyl groups from acetyl-CoA to arylamines. This enzyme has broad specificity for aromatic amines, particularly serotonin.
f. **Nd6 (NADH-ubiquinone oxidoreductase chain 6)**: Nd6 is a core subunit of the mitochondrial membrane respiratory chain NADH dehydrogenase (Complex I), which functions in the transfer of electrons from NADH to the respiratory chain. Defects in MT-ND6 are a cause of Leber hereditary optic neuropathy (LHON), a maternally inherited disease resulting in optic nerve dysfunction and loss of central vision.
g. **Nrn1 (neuritin 1)**: Nrn1 promotes neurite outgrowth and arborization of neuritic processes in primary hippocampal and cortical cells. It is expressed in postmitotic-differentiating neurons and neuronal structures associated with neural plasticity. The expression of this gene can be induced by neural activity and neurotrophins.
h. **Ptgs2 (Prostaglandin-endoperoxide synthase 2)**: Ptgs2 is the key enzyme in prostaglandin biosynthesis. It also functions as a major mediator of inflammation and/or a role for prostanoid signaling in activity-dependent plasticity. Expression Ptgs2 can be induced by cytokines and mitogens, which is responsible for the prostanoid biosynthesis involved in inflammation and mitogenesis, respectively.
i. **Scube1 (Signal peptide, CUB domain, EGF-like 1)**: Scube1 functions as an adhesive molecule. It is expressed in platelets and endothelial cells and its matrix bound and soluble fragments may play a critical role in vascular biology. Scube1 is down-regulated by inflammatory cytokines.

Table 8: Selected Late-Response Genes Following Sarin Treatment.

Gene Symbol*	Time Point (Hr)	Expression Level (Control)**	Post-Treatment Fold Change***			
			Control	0.4xLD50	0.6xLD50	0.75xLD50
Klk8	48	285.41	1.00	-1.05	-1.19	1.34
	120		1.00	1.31	1.20	1.56
Nr4a2	120	639.36	1.00	1.33	1.57	1.51
	600		1.00	1.64	1.73	1.87

*Genes that first showed significant expression changes at or after 48 hours following sarin treatment.
**Average value of the relative expression levels of controls at the corresponding time points.
***Negative fold change indicates down-regulation.

A brief description of the presently known functions of these late-response genes is shown below:

a. **Klk8 (Kallikrein-related peptidase 8)**: Klk8 is a serine protease that is capable of degrading a number of proteins such as fibrinogen, kininogen, fibronectin and collagen type IV. It cleaves L1CAM (L1 cell adhesion molecule) in response to increased neural activity. Klk8 induces neurite outgrowth and fasciculation of cultured hippocampal neurons. It plays a role in the formation and maturation of orphan and small synaptic boutons in the Schaffer-collateral pathway and regulates Schaffer-collateral long-term potentiation in the

hippocampus required for memory acquisition and synaptic plasticity. It also has a role in the secondary phase of pathogenesis following spinal cord injury. Klk8 is increased >10-fold in the hippocampus from patients with Alzheimer disease compared to controls.

b. **Nr4a2 (Nuclear receptor subfamily 4, group A, member 2)**: Nr4a2 is a member of the steroid-thyroid hormone-retinoid receptor superfamily. It functions as a general coactivator of gene transcription. Mutations in this gene have been associated with disorders related to dopaminergic dysfunction, including Parkinson disease, schizophrenia, and manic depression. Nr4a2 is rapidly and transiently expressed after cell activation, during the G0-G1 transition of the cell cycle. However, expression of Nr4a2 is not mandatorily associated with cell cycle.

Table 9: Selected Persistent Genes Following Sarin Treatment.

Gene Symbol*	Time Point (Hr)	Expression Level (Control)**	Fold Change (Post-treatment)***			
			Control	0.4xLD50	0.6xLD50	0.75xLD50
Dusp5	6	279.29	1.00	1.03	1.39	1.06
	12		1.00	1.11	1.35	1.29
	24		1.00	1.43	1.59	1.56
	48		1.00	1.31	1.12	1.37
	600		1.00	1.35	1.39	1.49
Egr1	2	1103.11	1.00	-1.05	-1.13	1.44
	12		1.00	1.27	1.38	1.72
	24		1.00	1.05	1.00	1.55
	120		1.00	1.33	1.43	1.51
	600		1.00	1.28	1.21	1.11
Egr2	6	145.72	1.00	1.14	1.58	1.30
	12		1.00	1.13	1.18	1.44
	24		1.00	1.14	1.27	1.36
	600		1.00	1.48	1.43	1.16
Egr3	6	548.96	1.00	-1.12	1.13	1.24
	12		1.00	-1.02	1.03	1.36
	24		1.00	-1.05	1.00	1.56
	120		1.00	1.11	1.16	1.30
Epha6	6	180.83	1.00	1.50	1.24	1.48
	12		1.00	1.12	1.22	1.56
	48		1.00	1.06	-1.08	1.36
	600		1.00	1.10	1.49	1.43
Inhba	6	210.72	1.00	1.07	1.23	1.54
	12		1.00	-1.19	-1.39	1.68
	24		1.00	1.11	1.12	1.70
	48		1.00	-1.07	-1.12	1.55
	120		1.00	1.05	1.23	1.31
	600		1.00	1.12	1.14	1.34
Per1	2	384.00	1.00	-1.14	1.01	1.44
	6		1.00	-1.08	1.10	1.31
	120		1.00	1.18	1.34	1.35
	600		1.00	1.19	1.31	1.34

Rasd1	2	220.04	1.00	1.02	1.11	1.56
	12		1.00	1.16	1.34	1.58
	24		1.00	1.25	1.29	1.71
	120		1.00	1.30	1.28	1.53
Tnfrsf25	2	129.25	1.00	-1.12	-1.26	-1.41
	48		1.00	1.23	1.27	1.36
	600		1.00	1.25	1.40	1.39

*Genes showed persistent expression changes (i.e., from early to late time points) after sarin treatment.
**Average value of the relative expression levels of controls at the corresponding time points.
***Negative fold change indicates down-regulation.

A brief description of the presently known functions of these persistently altered genes is shown below:

a. **Dusp5 (Dual specificity phosphatase 5)**: Dusp5 belongs to the protein-tyrosine phosphatase family, non-receptor class dual specificity subfamily. Dusp5 inactivates its target kinase by dephosphorylating both the phosphoserine/threonine and phosphotyrosine residues. It negatively regulates members of the mitogen-activated protein kinase superfamily, which are associated with cellular proliferation and differentiation. Dusp5 displays highest phosphatase activity toward ERK1 (Extracellular signal-regulated kinase 1). Dusp5 is localized in the nucleus and is highly expressed in brain.
b. **Egr1 (Early growth response 1)**: Egr1 functions as a transcriptional regulator. It activates the transcription of target genes that are required for mitogenesis and differentiation. Expression of Egr1 is induced by growth factors.
c. **Egr2 (Early growth response 2)**: Egr2 is a transcription factor. It binds to two specific DNA sites located in the promoter region of Hoxa4 (homeobox A4), a transcription factor that plays a role in the regulation of gene expression, morphogenesis, and differentiation. Egr2 interacts with HCFC1 (host cell factor C1), a protein involved in transcriptional regulation and cell cycle control during herpes simplex virus infection. Defects in Egr2 are associated with several diseases caused by hypomyelination neuropathy.
d. **Egr3 (Early growth response 3)**: Egr3 functions as a transcription factor. It is an immediate-early growth response gene which is induced by mitogenic stimulation. Egr3 participates in the transcriptional regulation of genes in controlling biological rhythm. It also has a role in a wide variety of processes such as negative regulation of apoptosis, endothelial cell growth and migration, and neuronal development.
e. **Epha6 (Eph receptor A6)**: Epha6, which belongs to the receptor tyrosine kinase family, is a receptor for members of the ephrin-A family. It binds GPI-anchored ephrin-A family ligands residing on adjacent cells, leading to contact-dependent bidirectional signaling into neighboring cells. The signaling pathway downstream of the receptor is referred to as forward signaling while the signaling pathway downstream of the ephrin ligand is referred to as reverse signaling. Epha6 is involved in axon guidance.
f. **Inhba (Inhibin beta-A)**: Inhibins are involved in regulating a number of diverse functions such as hypothalamic and pituitary hormone secretion, germ cell development and maturation, and nerve cell survival. Inhba is involved in cell cycle arrest and defense response.
g. **Per1 (Period homolog 1 (Drosophila))**: Per1 is a component of the circadian clock mechanism, which is essential for generating circadian rhythms. It functions as a negative

element in the circadian transcriptional loop. Per1 influences clock function by interacting with other circadian regulatory proteins and transporting them to the nucleus. Phosphorylation of Per1 by CSNK1 (casein kinase 1) leads to its cytoplasmic retention and subsequent ubiquitination and degradation. Per1 is phosphorylated upon DNA damage, probably by ATM (ataxia telangiectasia mutated) or ATR (ataxia telangiectasia and Rad3 related). Per1 negatively regulates transactivation induced by Clock (circadian locomotor output cycles kaput) / Npas2 – ARNTL (aryl hydrocarbon receptor nuclear translocator-like)/ARNTL2 (aryl hydrocarbon receptor nuclear translocator-like 2).

h. **Rasd1 (RAS, dexamethasone-induced 1)**: Rasd1 is a small GTPase. It negatively regulates the transcription regulation activity of the APBB1 (amyloid beta (A4) precursor protein-binding, family B, member 1) – APP (amyloid beta (A4) precursor protein) complex via its interaction with APBB1. Rasd1also forms a ternary complex with Nos1ap (nitric oxide synthase 1 (neuronal) adaptor protein) and NOS1 (nitric oxide synthase 1, neuronal). Rasd1 may play a role in dexamethasone-induced alterations in cell morphology, growth and cell-extracellular matrix interactions.

i. **Tnfrsf25 (Tumor necrosis factor receptor superfamily, member 25)**: Tnfrsf25 is a member of the TNF-receptor superfamily. Tnfrsf25 functions as a receptor for Tnfsf12 (tumor necrosis factor (ligand) superfamily, member 12). Tnfrsf25 interacts strongly via the death domains with Tnfrsf1 (tumor necrosis factor receptor superfamily, member 1) and Tradd (Tnfrsf1a-associated via death domain) to activate at least two distinct signaling cascades, apoptosis and NFκB (nuclear factor of kappa light polypeptide gene enhancer in B-cells) signaling pathway. Tnfrsf25 also interacts with Bag4 (Bcl2-associated athanogene 4), a member of the Bag1 antiapoptotic protein family.

3.4 Sarin-Induced Hippocampal Pathway Perturbation

Pathway perturbation at each time point following sarin treatment was determined by search the biological annotation databases with the differentially expressed genes as input data. Statistically significant enrichment of differentially expressed genes in specific pathways and was interpreted as differential pathway modulation. Specifically, up-regulated pathways were identified using the list of up-regulated genes as input data, whilst the down-regulated pathways were identified using the list of down-regulated genes as input data. In addition, the up- and down-regulated genes were combined and used as input date in the analysis to identify pathways that have significant enrichment of differentially expressed genes. The complete lists of sarin-induced pathway perturbation all eight time points are shown in Supplemental Table 5, A-H.

The results of pathways perturbation at all time points were merged into a single file. Pathways were then categorized according to their biological functions. The results of pathway perturbation in four major functional classes, gene/protein expression, signaling pathways, defense/repair/cell death and neuronal/behavioral/cognitive functions, are shown in Tables 10-18.

3.4.1 Gene Expression Process

Differential modulation of pathways involved in the gene expression process (including gene transcription, mRNA processing, mRNA transport, translation, post-translation modifications and protein transport) are shown in Tables 10-12.

3.4.1.1 Transcriptional and Posttranscriptional Regulations

Sarin treatment induced up-regulation of genes involved in transcription at multiple time points, especially 6 hours, 1-5 days and 25 days post-treatment (see Table 10). The increased gene transcription at 5 and 25 days and could be the result of down-regulation of transcription repressors/co-repressors at the 2-day time point. In addition, changes in the chromatin structures, a result of increased histone methylation and acetylation, likely played a role in the modulation of gene transcription as well. Although mRNA processing and transport also showed up-regulation at multiple time points, they do not completely coincide with gene transcription. For instance, up-regulation of mRNA processing occurred as early as 2 hours after sarin treatment, while up-regulation of transcription was only observed at the 6-hour time point. However, up-regulation of mRNA processing at later time points generally follows up-regulation of gene involved in transcription.

Table 10: Perturbation of Pathways Involved in Transcriptional and Posttranscriptional Regulations in Hippocampus Following Sarin Treatment.[1, 2]

Transcription regulation, mRNA/ncRNA processing and transport	Time Point (Hr)							
	2	6	12	24	48	120	240	600
transcription								
transcription regulation								
positive regulation of transcription								
negative regulation of transcription								
regulation of transcription from RNA polymerase II promoter								
positive regulation of transcription from RNA polymerase II promoter								
negative regulation of transcription from RNA polymerase II promoter								
transcription cofactor activity								
transcription repressor activity								
transcription corepressor activity								
chromosome organization								
chromatin								
chromatin modification								
chromatin remodeling complex								
chromatin regulator								
histone modification								
histone methylation								
histone acetylation								
histone acetyltransferase complex								
mRNA processing								
ncRNA processing								
RNA splicing								
Spliceosome								
regulation of mRNA stability								
mRNA transport								

[1]Statistically significant enrichment of differentially expressed genes in specific pathways was interpreted as pathway perturbation.

[2]Color scheme: red – enrichment of up-regulated genes; red and green – enrichment of both up- and down-regulated genes; green – enrichment of down-regulated genes; black– enrichment of differentially expressed genes.

3.4.1.2 Translational and Posttranslational Regulations

Translation, especially translation initiation, was also up-regulation at several time points following sarin treatment (see Table 11). Although the ribosomal subunits/proteins were strongly up-regulated at the 5-day time point, sarin treatment induced down-regulation of these genes at 2 hours post-treatment. The genes involved in protein transport showed a similar pattern of up-regulation (i.e., up-regulation at three time points). However, they were down-regulated at other time points. Protein folding and protein complex assembly were minimally affected at early time points; they showed up-regulation at the last time point 25 days.

Table 11: Perturbation of Pathways Involved in Translational and Posttranslational Regulations in Hippocampus Following Sarin Treatment.[1, 2]

Translation egulation, post-translational modifications, protein transport	Time Point (Hr)							
	2	6	12	24	48	120	240	600
translation								
regulation of translation								
negative regulation of translation								
translational initiation								
regulation of translational initiation								
regulation of eIF4e and p70 S6 Kinase								
translation initiation factor activity								
translation elongation factor activity								
ribonucleoprotein complex								
ribosome								
ribosomal protein								
ribosomal subunit								
large ribosomal subunit								
protein localization								
regulation of protein localization								
establishment of protein localization								
protein transport								
regulation of protein transport								
protein localization in organelle								
protein localization in nucleus								
protein import								
protein import into nucleus								
protein targeting								
protein folding								
molecular chaperone								
posttranslational modification								
glycoprotein biosynthetic process								
protein complex assembly								
regulation of protein complex assembly								
positive regulation of protein complex assembly								
regulation of protein complex disassembly								
negative regulation of protein complex disassembly								

[1]Statistically significant enrichment of differentially expressed genes in specific pathways was interpreted as pathway perturbation.

[2]Color scheme: red – enrichment of up-regulated genes; red and green – enrichment of both up- and down-regulated genes; green – enrichment of down-regulated genes; black – enrichment of differentially expressed genes.

3.4.1.3 Endomembrane System, Protein Folding and Ubiquitin-Mediated Proteolysis

The results shown above suggested that sarin treatment strongly stimulated transcription and translation. It was quite unexpected that the endoplasmic reticulum (ER) and Golgi apparatus, the subcellular organelles essential for processing (e.g., folding, post-translational modifications and packaging) the newly synthesized proteins, was strongly down-regulated in almost all time points analyzed in this study (except the last time point). Consistent with the down-regulation of the endomembrane system, up-regulation of ER overload, unfolded protein stress and ER stress was observed at the 5-day time point, which preceded the massive down-regulation of gene expression that occurred at 10 days post-treatment (see Table 12). In addition, the ubiquitin-mediated proteolysis was strongly up-regulated at five time points. This is probably the consequences of the dysregulation of the endomembrane system and the resulting ER overload/stress.

Table 12: Perturbation of Pathways Involved in Endomembrane System, Protein Folding and Ubiquitin-Mediated Proteolysis in Hippocampus Following Sarin Treatment.[1, 2]

Endomembrane system & ubiquitin-mediated proteolysis	Time Point (Hr)							
	2	6	12	24	48	120	240	600
endomembrane system								
nuclear envelope-endoplasmic reticulum network								
endoplasmic reticulum								
ER-Golgi transport								
Golgi apparatus								
trans-Golgi network								
vesicle								
vesicle-mediated transport								
Golgi vesicle transport								
nucleocytoplasmic transport								
unfolded protein response								
ER overload response								
response to endoplasmic reticulum stress								
ER-nuclear signaling pathway								
cellular protein catabolic process								
proteolysis involved in cellular protein catabolic process								
ER-associated protein catabolic process								
ubiquitin-dependent protein catabolic process								
proteasomal ubiquitin-dependent protein catabolic process								
ubl conjugation								
ubl conjugation pathway								
regulation of protein ubiquitination								
ubiquitin mediated proteolysis								
proteasome / proteasome complex								

[1]Statistically significant enrichment of differentially expressed genes in specific pathways was interpreted as pathway perturbation.
[2]Color scheme: red – enrichment of up-regulated genes; green – enrichment of down-regulated genes; black – enrichment of differentially expressed genes.

3.4.2 Signaling Pathways

The signaling pathways essential for neuronal activities, neurotrophin signaling and Wnt signaling, were up-regulated at multiple time points (see Table 13). Although the TGF-beta signaling pathway is not neural specific, it has broad functions in the regulation of cell proliferation, differentiation, adhesion, migration in many cell types, including neuronal cells. Up-regulation of this pathway is therefore not unexpected. The ErbB, VEGF, Gonadotropin-releasing hormone (GnRH) and mTOR signaling pathways also showed alteration/up-regulation following sarin treatment. Since the MAP kinase pathway is the common down-stream signaling pathway for most of these pathways, it is up-regulated in almost all time points.

Table 13: Perturbation of Signaling Pathways in Hippocampus Following Sarin Treatment.[1, 2]

Signaling Pathways	Time Point (Hr)							
	2	6	12	24	48	120	240	600
TGF-beta signaling pathway								
Neurotrophin signaling pathway								
Wnt signaling pathway								
ErbB signaling pathway								
VEGF signaling pathway								
GnRH signaling pathway								
mTOR signaling pathway								
enzyme linked receptor protein signaling pathway								
transmembrane receptor protein tyrosine kinase signaling pathway								
small GTPase mediated signal transduction								
MAPK signaling pathway								
negative regulation of MAP kinase activity								
Phosphatidylinositol signaling system								

[1]Statistically significant enrichment of differentially expressed genes in specific pathways was interpreted as pathway perturbation.

[2]Color scheme: red – enrichment of up-regulated genes; red and green – enrichment of both up- and down-regulated genes; green – enrichment of down-regulated genes; black – enrichment of differentially expressed genes.

3.4.3 DNA Damage/Repair, Glutathione Metabolism, Apoptosis and Tissue Repair

Sarin induces lethality mostly by inhibition of AChE and cholinergic overstimulation. Its effect on damage of subcellular organelles and other constituents is not well understood. The results in Table 14 strongly suggested that sarin exposure likely resulted in DNA damage, as the pathways involved in DNA repair were up-regulated at 6 hours after sarin treatment. Several members of the glutathione S-transferase (GST) family were down-regulated at two time points, 12 hours and 10 days, after sarin treatment. One of the functions of GST is to catalyze the conjugation of reduced glutathione to toxins, including (but not limited to) free radicals and reactive oxygen species, thereby detoxifying these compounds. In addition, GSTs could also bind to toxins and function as transport proteins. Down-regulation of such an important cellular defense system will likely further exacerbate the toxicity of sarin treatment, especially its DNA-damaging effects. The consequences of these events are thus likely the induction of apoptosis (and up-regulation of apoptosis regulation), which occurred three times (each covered two time points), with two of them occurring immediately after the GSTs down-regulation. Interestingly, genes

involved in the negative regulation of apoptosis were up-regulated at two time points, with one occurring at the later phase of the second peak of apoptosis. To counter these adverse events and re-establish a homeostatic state, pathways for repairing sarin-induced injury were activated. These include angiogenesis and wound healing.

Table 14: Perturbation of Pathways Involved in DNA Damage/Repair, Glutathione Metabolism, Apoptosis and Tissue Repair in Hippocampus Following Sarin Treatment.[1, 2]

DNA damage/repair, glutathione metabolism, stress, apoptosis & tissue repair (including angiogenesis)	Time Point (Hr)							
	2	6	12	24	48	120	240	600
DNA damage								
DNA repair								
response to DNA damage stimulus								
cellular response to stress								
glutathione metabolism								
glutathione S-transferase, N-terminal								
glutathione S-transferase, C-terminal								
glutathione S-transferase, C-terminal-like								
glutathione S-transferase/chloride channel, C-terminal								
glutathione S-transferase, Mu class								
metabolism of xenobiotics by cytochrome P450								
apoptosis								
regulation of apoptosis								
positive regulation of apoptosis								
activation of caspase activity by cytochrome c								
negative regulation of apoptosis								
negative regulation of neuron apoptosis								
wound healing								
vasculature development								
blood vessel morphogenesis								
angiogenesis								
regulation of angiogenesis								

[1]Statistically significant enrichment of differentially expressed genes in specific pathways was interpreted as pathway perturbation.
[2]Color scheme: red – enrichment of up-regulated genes; green – enrichment of down-regulated genes; black – enrichment of differentially expressed genes.

3.4.4 Neuronal Growth, Neuron Projection, Synaptic Structure, Synaptic Plasticity and Behavioral/Cognitive Functions

The genes in this category are among the most up-regulated genes. Additionally, they showed up-regulated at multiple time points. For instance, genes involved in neurogenesis and regulation of neurogenesis were up-regulated from 12 hours to 5 days following sarin treatment. However, up-regulation of genes involved in neuron differentiation and morphogenesis mainly occurred as a single peak at 48 hours post-treatment (Table 15).

Table 15: Perturbation of Pathways Involved in Neuronal Growth and Differentiation in Hippocampus Following Sarin Treatment.[1, 2]

Neuronal growth & differentiation	Time Point (Hr)							
	2	6	12	24	48	120	240	600
regulation of cell proliferation								
positive regulation of cell proliferation								
negative regulation of cell proliferation								
anti-proliferative protein								
regulation of growth								
cell proliferation in forebrain								
neurogenesis								
regulation of neurogenesis								
mitotic cell cycle								
regulation of cell cycle								
cell cycle arrest								
mitotic sister chromatid segregation								
positive regulation of cell differentiation								
negative regulation of cell differentiation								
neuron differentiation								
regulation of neuron differentiation								
cell morphogenesis involved in differentiation								
cell morphogenesis involved in neuron differentiation								
neuron development								
neuron maturation								
regulation of cell size								
positive regulation of cell size								

[1]Statistically significant enrichment of differentially expressed genes in specific pathways was interpreted as pathway perturbation.
[2]Color scheme: red – enrichment of up-regulated genes; green – enrichment of down-regulated genes; black – enrichment of differentially expressed genes.

Coincident with (or immediately following) the activation of the neuronal differentiation pathways, up-regulation of pathways with essential roles in neuron projection morphogenesis, axon guidance, axon extension, axon ensheathment and myelination was observed (Table 16). Unlike the pathways involved in the axonal morphogenesis, pathways with essential roles in dendritic arborization and synaptic structure were broadly up-regulated at almost all time points after sarin treatment.

Table 16: Perturbation of Pathways Involved in Neuron Projection, Axonal, Dendritic and Synaptic Structure in Hippocampus Following Sarin Treatment.[1, 2]

Neuron projection, synaptic structures,	Time Point (Hr)							
	2	6	12	24	48	120	240	600
neuron projection								
neuron projection development								
regulation of cell morphogenesis								
cellular component morphogenesis								
neuron projection morphogenesis								
regulation of neuron projection development								
axonogenesis								
regulation of axonogenesis								
positive regulation of axonogenesis								
axon guidance								
regulation of axon extension								
positive regulation of axon extension								
axon								
axon ensheathment								
myelination								
dendrite								
dendrite development								
regulation of dendrite development								
dendritic shaft								
dendritic spine								
synapse								
synapse part								
synapse organization								
synaptogenesis								
presynaptic membrane								
synaptosome								
synaptic vesicle								
postsynaptic membrane								
postsynaptic density								
ionotropic glutamate receptor, AMPA/kainate types								

[1]Statistically significant enrichment of differentially expressed genes in specific pathways was interpreted as pathway perturbation.
[2]Color scheme: red – enrichment of up-regulated genes; green – enrichment of down-regulated genes; black – enrichment of differentially expressed genes.

As the pathways for neuronal projection, axonal morphogenesis, dendritic arborization and synaptic structure were strongly activated, synaptic transmission (including postsynaptic membrane potential) and synaptic plasticity (e.g. long-term potentiation) were also broadly up-regulated (Table 17).

Table 17: Perturbation of Pathways Involved in Synaptic Transmission and Synaptic Plasticity in Hippocampus Following Sarin Treatment.[1, 2]

Synaptic transmission, membrane potential	Time Point (Hr)							
	2	6	12	24	48	120	240	600
transmission of nerve impulse								
regulation of transmission of nerve impulse								
positive regulation of transmission of nerve impulse								
synaptic transmission								
nerve-nerve synaptic transmission								
synaptic transmission, glutamatergic								
regulation of synaptic transmission								
positive regulation of synaptic transmission								
regulation of membrane potential								
regulation of postsynaptic membrane potential								
regulation of excitatory postsynaptic membrane potential								
regulation of action potential								
regulation of action potential in neuron								
regulation of synaptic plasticity								
regulation of neuronal synaptic plasticity								
regulation of long-term neuronal synaptic plasticity								
Long-term potentiation								
Long-term depression								

[1]Statistically significant enrichment of differentially expressed genes in specific pathways was interpreted as pathway perturbation.
[2]Color scheme: red – enrichment of up-regulated genes; green – enrichment of down-regulated genes; black – enrichment of differentially expressed genes.

Similar to the pathways involved in synaptic transmission and synaptic plasticity, pathways related to behavioral and cognitive functions (e.g., learning and memory) were also up-regulated at multiple time points (see Table 18). This is expected; in fact these behavioral and cognitive functions are supported by neurological function such as synaptic transmission and synaptic plasticity. Interestingly, the pathways related to several neurodegenerative diseases were up-regulated at the 5-day time point, which precedes the massive gene depression in hippocampus that occurred at the 10-day time point.

Table 18: Perturbation of Pathways Involved in Behavioral/Cognitive Functions and Neurodegenerative Diseases in Hippocampus Following Sarin Treatment.[1, 2]

Behavioral and cognitive functions & neurodegenerative diseases	Time Point (Hr)							
	2	6	12	24	48	120	240	600
behavior								
locomotory behavior								
visual behavior								
learning or memory								
learning								
memory								
visual learning								
fear response								
sensory perception of pain								
Alzheimer's disease								
Huntington's disease								
Parkinson's disease								
Amyotrophic lateral sclerosis (ALS)								

[1]Statistically significant enrichment of differentially expressed genes in specific pathways was interpreted as pathway perturbation.
[2]Color scheme: red – enrichment of up-regulated genes; black – enrichment of differentially expressed genes.

4. DISCUSSION

Sublethal sarin exposure induced prolonged impairment of cognitive and neurobehavioral functions (Kassa et al, 2001a; Kassa et al, 2001b; Hulet et al, 2002; Kassa et al, 2004; Grauer et al, 2008). For instance, mice exposed to an asymptomatic dose of sarin display long-lasting deficiency in emotional learning (Lucot, personal communication). These observations suggested that traditional methods for assessing sarin toxicity lack the sensitivity to detect the changes associated with organ injury. Therefore, the goal of this study is to investigate the effects of sublethal sarin exposure on gene expression, based on the rationale that the gene expression changes could serve as sensitive indicators of sarin-induced injury. Additionally, pathway perturbation, resulted from gene expression changes, could provide novel insights into the molecular mechanism(s) of sarin-induced organ injury.

Three doses of sarin, 0.4x, 0.6x, and 0.75x LD50, were used in this study. The results in Figure 2 show that while the two lower doses are asymptomatic or mildly symptomatic (based on functional observations), the highest dose, 0.75x LD50, caused observable signs of sarin intoxication. Therefore, the dose range employed in this study provided a good coverage for sarin-induced injury.

Compared to functional observation assessment, analysis of AChE inhibition in both brain and blood appeared to be more sensitive in detecting sarin toxicity, as significant (although relatively small) decreases in AChE activities were observed in some animals treated with the lowest dose, 0.4x LD50. Consistent with this notion, most of the animals treated with sarin at 0.6x LD50 showed a significant inhibition of AChE in frontal lobe. Blood AChE seemed to be highly sensitive to sarin exposure, as the lowest dose (0.4x LD50) caused significant AChE inhibition

up to 12 hours post-treatment. However, the enzyme activity quickly recovered after this time points even in the animals treated with 0.6x and 0.65x LD50. Analysis of BuChE activity seemed to be rather insensitive in detecting sarin exposure, as most animals treated with the highest dose, 0.75xLD50, did not show statistically significant inhibition.

Although six brain regions and six peripheral organs have been collected from control and sarin-treated animals, funding received only allows the analysis of one brain region (or organ). Hippocampus is one of the most sensitive brain regions to sarin-induced injury. For instance, neural injury in the dentate gyrus is detectable very early after sarin treatment (Lucot, personal communication). Additionally, this brain region plays a critical role in cognitive functions, such as learning and memory. Decline in memory performance, including impairment of working and reference memory processes, has been reported to be the major long-lasting effects resulted from sublethal sarin exposure (Hatta et al., 1996; Yokoyama et al. 1998; Kassa et al, 2001a; Kassa et al, 2001b; Kassa et al, 2004). Because of these reasons, hippocampus was chosen as the first sample set for the investigation of sarin-induced differential gene expression and pathway perturbation.

Gene expression profiling revealed that sarin treatment resulted in differential expression of a large number of genes - from several hundred to approximately two thousand genes, dependent on the time after the treatment. The numbers of differentially expressed genes are significantly higher than expected. This is probably due to the fact that an exon array, which contains probe sets for >300,000 transcripts (including alternatively spliced products), was used in this study. Although a filter for eliminating low-quality probes was implemented during data analysis, expression profiles of ~129,000 transcripts nevertheless were obtained. Compared to the expression profiles of ~25,000 transcripts, this dataset is thus significantly richer than that obtained using microarray based on probe sets designed around the 3' end of the mRNAs. Additionally, the option of all pair-wise comparison was selected during data analysis. This option allowed the identification of not only the genes showing sarin-induced differential expression, but also the genes showing differential dose response.

SOM clustering analysis is a very powerful pattern recognition technique for identifying gene groups with unique dose response. Using this technique, the seemingly complex patterns of sarin-induced differential gene expression could be effectively separated into three categories, dose-dependent, dose independent and response with transitional changes. Based on the dose response pattern, the roles of these gene categories in the response to sarin toxicity can be estimated. For instance, dose-dependent genes are likely related to defense/repair, while dose independent genes are probably involved in toxic response. As such this scientific logic/conjecture is not definitive as the biological significance of these gene expression changes must be validated.

Detailed examination of the gene expression changes that occurred at early and late time points reveal an interesting pattern of temporal response of sarin-induced differential gene expression. At early time points, a significant number of genes encoding for transcription activator (e.g. Npas4), or signaling pathways regulators (e.g. Ddit4) with gene expression as downstream targets, were up-regulated. These changes likely set the stage of large scale differential gene expression at later time points.

The observation of Nptx2 up-regulation at early time points is of particular interest. Moran et al (2008) reported Nptx2 protein was increased in PD tissues and suggested that it may be involved in the pathology of this disease. A number of investigators have shown that OP exposure induced PD-like symptoms, which might be mechanistically related to alterations in the dopaminergic system (Karen et al, 200; Choudhary et al, 2002; Masoud et al, 2011; Torres-Altoro et al, 2011). However, the result reported here that Nptx2 was up-regulated after sarin treatment provides an alternative mechanism for OP-induced PD-like symptoms.

At the later time points, the toxic effect of sarin emerged, as evidenced by the up-regulation of genes involved in neural inflammation and apoptosis (e.g. Ptgs2 and Acvr1c). Consistent with this observation, other investigators also reported that sarin (and soman) exposure that caused no overt clinical signs resulted in an inflammatory response, as indicated by the increases in brain mRNAs for interleukin (IL)-1β, tumor necrosis factor-α, and IL-6 (Svensson et al., 2001; Henderson et al, 2002). At these later time points, proteolysis was initiated to clear the damaged proteins (e.g. up-regulation of Hectd2).

Considering the large number of genes that showed differential expression at the 25-day time point, it is quite unexpected that the number of late response genes is relatively small. Close examination revealed that most of the genes that are differentially expressed at late time points also showed expression changes at early time points. In other words, these genes showed persistent expression changes from early to late time points. Interestingly, transcription regulators are quite common among these persistently altered genes (e.g. Egr1, Egr2 and Egr3). Receptors and signaling pathway regulators, with roles in neuronal morphology, cell cycle regulation, defense response and apoptosis, are also common.

Despite that these results provided interesting information on gene expression changes following sarin exposure, they provided limited insight into the mechanism of sarin-induced delay neurotoxicity as reported in the literature (Husain et al, 1993; Nishiwaki et al, 2001; Miyaki et al, 2005). To address this issue, analysis of pathway perturbation was thus conducted. Consistent with the result of temporal gene expression analysis, sarin treatment appears to activate gene transcription pathways at multiple time points, especially 6 hours, 1, 2, 5 and 25 days post-treatment. To facilitate gene transcription, coordinated changes in chromatin structures (resulted from histone methylation/acetylation), mRNA processing/transport were also observed. Increased gene transcription following sarin treatment is expected, as it is essential for almost all cellular responses, including defense, repair and toxic responses, to exogenous insults/stressors. As translation is also an essential process for almost all defense, repair and toxic responses to exogenous insults/stressors, it is also strongly up-regulated following sarin treatment. In addition, the pathways related to protein transport showed a similar pattern of activation. Taken together, these results suggested that after sarin treatment, cells strongly activated the genes expression process to counter the adverse effects of sarin toxicity.

However, successful defense against sarin toxicity and repair of sarin-induced injury require not only protein synthesis, but also proper processing (such as folding and post-translational modifications) of the newly synthesized proteins and transporting the processed proteins to the subcellular sites, in which they perform their functions. The endomembrane system (including

the ER and the Golgi apparatus) is critical for protein processing, packaging and transport. Down-regulation of the ER and the Golgi apparatus at multiple time points after sarin treatment strongly suggested the newly synthesized proteins were not processed or transported out of the ER/Golgi apparatus, resulting in the accumulation of unprocessed/unfolded proteins in ER and subsequently ER overload and ER stress. This creates a scenario that on one hand, the sarin-induced damage could not be repaired, and on the other hand, the injured cells are under additional stress due to accumulation of unfolded proteins in ER. The combined effects of persistent presence of unrepaired damage and ER stress will likely trigger and perhaps even accelerate cell death.

Although sarin lethality through inhibition of AChE and cholinergic overstimulation is well established, the mechanism of sarin cellular toxicity is not completely clear, nor the effect of sarin on DNA damage. Klein et al (1987) reported that no increase in unscheduled DNA synthesis was observed in sarin-treated hepatocytes. On the contrary, significant decreases in DNA repair synthesis were detected. Based on these results, these investigators concluded that sarin may either inhibit DNA repair or protect DNA from damage. However, a more recent study showed that repeated low-dose (0.2x and 0.4x LD50) sarin exposure resulted in a significant increase in DNA fragmentation in leukocytes (Dave et al, 2007). However, a systematic dose-dependent response was not observed in parietal cortex, although DNA fragmentation did occur in some cases. The results of pathway analysis reported here strongly suggested that sarin likely caused DNA damage, as pathways related to DNA repair were up-regulated at 6 hour post-treatment. Besides DNA damage, sarin treatment also activated other stress response, as well as apoptosis (which can be considered as the "ultimate, uncompromised" stress response), at several time points. It should be noted that up-regulation of genes involved in the apoptosis pathway does not necessarily means that the apoptotic process is in fact executed. However, activation of pathways involved in tissue repair, such as wound healing and vasculature development, suggested the occurrence of sarin-induced injury, regardless the mechanisms of cell death (e.g. necrosis, apoptosis or both). Given the activation of stress response after sarin treatment, it is quite expected that several members of the glutathione S-transferase (GST) family were down-regulated at two time points after sarin treatment. Down-regulation of the GST defense system will likely further exacerbate the adverse effects of sarin toxicity.

Signaling pathways serve as the communication network in the cells. For instance, activation of VEGF signaling pathway results in angiogenesis, a tissue repair pathway that was up-regulated as described above. Similarly, activation of the neurotrophin, Wnt, TGF-beta and ErbB signaling pathways, which can stimulate neuronal cells proliferation, differentiation and morphogenesis, is probably part of the repair process. Consistent with this premise, up-regulation of pathways related to neuronal growth/differentiation (e.g. neurogenesis, regulation of neurogenesis and neuronal differentiation), neuron projection development (e.g. neuron projection morphogenesis, axon guidance, axon ensheathment/myelination and dendritic arborization) and synapse organization (e.g. synaptogenesis, presynaptic membrane and postsynaptic density) was observed at multiple time points after sarin treatment. These structural changes could thus support the functional enhancement in synaptic transmission, synaptic plasticity (e.g. long-term potentiation and long-term depression) and cognitive functions (e.g., learning and memory). Interestingly, pathways related to several neurodegenerative diseases

were up-regulated at 5 days post-treatment, due to differential expression of genes involved in mitochondrial dysfunction. This result suggested that mitochondrion could also be an intracellular target of sarin. However, this has to be confirmed in future studies.

The results of pathway analysis at each time point provided a snapshot of the events following sarin treatment. Based on the sequence and the biological significance of these events, a model that aims to provide a mechanistic basis for sarin-induced hippocampal injury is proposed as outlined below.

1. Upon sarin exposure, cellular damage (e.g. DNA damage) occurred. As a result, the apoptotic pathway was activated. Proliferation pathway, as part of the repair process, was also activated. This response was supported by the processing of existing transcripts, rather than synthesis of new transcripts.

2. Cellular damage subsequently activated the DNA repair pathway and the clearance of the damaged proteins by proteolysis. Signaling pathways were activated to coordinate the cellular response. This includes the repair processes, which is supported by gene transcription, RNA processing/transport, translation, protein processing/transport. The newly transcribed genes are involved in proliferation, differentiation, neuronal projection, axonal, dendritic, and synaptic structures, neurological and cognition functions. However, down-regulation of ER and the Golgi apparatus occurred, resulting in dysfunction of the endomembrane system (such as dysregulation of the ER-Golgi transport and the trans-Golgi network). As the endomembrane system has a critical role in the processing of the newly synthesized proteins, damage to this subcellular organelle has significant consequences.

3. Signaling pathways that coordinate the repair process (including the gene/protein expression process, cell proliferation, differentiation, neuronal morphogenesis, and neurological and cognition functions) remained activated. However, the effects of sarin-induced cellular damage became more apparent. This includes not only dysfunction of the endomembrane system, but also the suppression of the GST defense system, which prevents the detoxification and removal of toxic compounds. Proteolysis for clearing the damaged proteins remained activated.

4. As sarin-induced injury persisted, signaling pathways that coordinate the repair process remained activated. However, apoptosis is activated in the cells that were unable to repair the damage. At the same time, pathways for tissue repair were also activated. Dysfunction of the endomembrane system became more significant, which prevented the newly synthesized proteins to be properly processed, correctly folded and transported to the target sites. As a result, the gene/protein expression that is to support the repair process remained highly activated. Persistent up-regulation of the gene/protein expression process and dysfunction of the endomembrane system resulted in the accumulation of unfolded proteins in the ER (i.e., ER overload), ER stress and subsequently cell death, which is associated with a broad spectrum of gene suppression at 10 days post-treatment.

5. However, this cycle of gene/protein expression – ER stress – cell death may repeat many times long after the exposure, as suggested by the similar patterns of gene expression and

pathway perturbation at 6 hours and 25 days. Such repeated cycle of neuronal cell death is likely the basis for sarin-induced delayed neurotoxicity and cognitive impairments as described in the literature (Husain et al, 1993; Nishiwaki et al, 2001; Miyaki et al, 2005).

5. CONCLUSION

In this study, differential gene expression in the hippocampal region was investigated in mice treated with sublethal doses of sarin using DNA microarray technology. Detailed interpretation of the microarray data using advanced bioinformatic techniques allows the identification of pathway modulation/perturbation in response of sarin-induced injury. Integration of these events, based on their biological significance and the temporal sequence of their occurrence, afforded a mechanistic model for sarin-induced delayed neurotoxicity. Testing/validation and overall accuracy of the developed model will require further studies and are the goals of future studies. Do note, all reference to changes in brain tissue is based upon mRNA data and major pathway effectors, and has not been fully referenced by protein content or protein activities data.

In addition to these accomplishments, it should also be emphasized that a number of brain regions and major peripheral organs from sarin-treated (and control) animals have been isolated and preserved. Analysis of gene expression changes and pathway perturbation using these specimens will provide a thorough understanding of sarin-induced differential gene expression, pathway perturbation and injury in these tissues. Integration of the results of this study and those that derived from future studies using the preserved tissues will reveal the molecular mechanism of sarin-induced injury at the organism level. This will significantly facilitate the efforts aiming to develop countermeasures against this chemical warfare agent.

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7. LIST OF SYMBOLS, ABBREVIATIONS, AND ACRONYMS

A_{260} – Absorbance at 260 nm
A_{280} – Absorbance at 280 nm
AChE - Acetylcholinesterase
AGCC – Affymetrix GeneChip Command Console
BuChE - Butyrylcholinesterase
DAVID - Database for Annotation, Visualization, and Integrated Discovery
DTRA - Defense Threats Reduction Agency
ER - Endoplasmic reticulum
FOB - Functional observational battery
GnRH - Gonadotropin-releasing hormone
GST - Glutathione S-transferase
IACUC - Institutional Animal Care and Use Committee
IL - Interleukin
LD50 - Mean Lethal Dose
OP - Organophosposphorus
PD - Parkinson's disease
RPM - Revolutions per minute
SOM - Self-organizing map
WSU - Wright State University
WT - Whole transcript

8. Appendix – Supplemental Data

Supplemental Table 1: FOB Scores of Control and Sarin-Treated Animals.[1, 2]

Observation Time Point (post-injection, Hr)	Sacrifice Time Point (post-injection)	Dose (xLD50)	FOB Score (average)	% of Max. Score (average)	St. Error
0.5	12 Hours	0	6.0	29%	0%
		0.4	7.6	36%*	1%
		0.6	7.7	37%*	1%
		0.75	13.0	62%*	5%
	2 Days	0	6.0	29%	0%
		0.4	6.0	29%	0%
		0.6	6.0	29%	0%
		0.75	15.5	74%*	4%
	5 Days	0	6.0	29%	0%
		0.4	6.0	29%	0%
		0.6	7.7	37%	2%
		0.75	15.8	75%*	2%
	10 Days	0	6.0	29%	0%
		0.4	6.0	29%	0%
		0.6	6.2	29%	0%
		0.75	17.6	84%*	1%
	25 Days	0	6.0	29%	0%
		0.4	6.4	30%	1%
		0.6	6.8	33%*	1%
		0.75	16.5	79%*	5%
1	2 Hours	0	6.0	29%	0%
		0.4	6.0	29%	0%
		0.6	7.0	34%	1%
		0.75	16.0	78%*	5%
	6 Hours	0	6.0	29%	0%
		0.4	6.0	29%	0%
		0.6	6.0	29%	0%
		0.75	14.0	67%*	4%
	24 Hours	0	6.0	29%	0%
		0.4	6.0	30%	1%
		0.6	6.0	29%	0%
		0.75	14.0	65%*	5%
	2 Days	0	6.0	29%	0%
		0.4	6.0	29%	0%
		0.6	6.0	29%	0%
		0.75	16.0	77%*	4%
	5 Days	0	6.0	29%	0%
		0.4	6.0	29%	0%
		0.6	8.0	37%	2%
		0.75	14.0	67%*	3%
	10 Days	0	6.0	29%	0%
		0.4	6.0	29%	0%
		0.6	6.0	30%	1%
		0.75	17.0	80%*	2%
	25 Days	0	6.0	29%	0%
		0.4	7.0	32%*	1%
		0.6	7.0	33%*	1%
		0.75	15.0	73%*	4%
4	6 Hours	0	6.0	29%	0%

		0.4	6.0	29%	0%
		0.6	6.0	29%	0%
		0.75	14.0	65%*	3%
	12 Hours	0	6.0	29%	0%
		0.4	6.0	30%	1%
		0.6	6.0	29%	0%
		0.75	11.0	52%*	3%
	24 Hours	0	6.0	29%	0%
		0.4	6.0	30%	1%
		0.6	6.0	29%	0%
		0.75	12.0	55%*	4%
	2 Days	0	6.0	29%	0%
		0.4	6.0	29%	0%
		0.6	6.0	29%	0%
		0.75	11.0	51%*	2%
	5 Days	0	6.0	29%	0%
		0.4	6.0	29%	0%
		0.6	6.0	29%	0%
		0.75	9.0	43%*	3%
	10 Days	0	6.0	29%	0%
		0.4	6.0	29%	0%
		0.6	6.0	30%	1%
		0.75	13.0	63%*	2%
	25 Days	0	6.0	29%	0%
		0.4	7.0	31%	1%
		0.6	7.0	35%*	1%
		0.75	9.0	42%*	1%
24	2 Days	0	6.0	29%	0%
		0.4	6.0	29%	0%
		0.6	6.0	29%	0%
		0.75	6.0	30%	0%
	5 Days	0	6.0	29%	0%
		0.4	6.0	29%	0%
		0.6	6.0	29%	0%
		0.75	6.0	29%	0%
	10 Days	0	6.0	29%	0%
		0.4	6.0	29%	0%
		0.6	6.0	29%	0%
		0.75	6.0	29%	0%
	25 Days	0	6.0	29%	0%
		0.4	6.0	29%	0%
		0.6	6.0	29%	0%
		0.75	6.0	29%	0%

[1]The FOB scores of the control and sarin-treated animals at 0.5, 1, 4 and 24 hours post-treatment are shown.
[2]Data of several experiments (2-hr, 6-hr and 24-hr time points for observation at 0.5 hour; 12-hr time point experiment for observation at 1 hour; 2-hr time point experiment for observation at 4 hours; 2-, 6-, 12- and 24-hour time point experiments for observation at 24 hours) were not available.
*Significant changes in observational functions ($p<0.05$).

Supplemental Table 2: Sarin-Induced Inhibition of AChE in Frontal Lobe.

Time Point	Sarin Dose	AChE Activity (nmoles/min/μL)		
		Average	St. Error	% Control
2 hours	Control	11.45	0.72	100%
	0.4x LD50	10.02	0.41	88%
	0.6x LD50	4.29	0.35	37%*
	0.75x LD50	2.17	0.16	19%*
6 hours	Control	10.32	0.72	100%
	0.4x LD50	8.70	0.22	84%
	0.6x LD50	5.46	0.14	53%*
	0.75x LD50	3.14	0.12	30%*
12 hours	Control	9.89	0.60	100%
	0.4x LD50	9.93	0.41	100%
	0.6x LD50	6.40	0.29	65%*
	0.75x LD50	3.24	0.16	33%*
24 hours	Control	11.19	0.11	100%
	0.4x LD50	9.84	0.16	88%*
	0.6x LD50	7.18	0.35	64%*
	0.75x LD50	3.79	0.18	34%*
48 hours	Control	11.40	0.35	100%
	0.4x LD50	9.24	0.36	81%*
	0.6x LD50	7.56	0.25	66%*
	0.75x LD50	4.24	0.21	37%*
120 hours	Control	10.88	0.30	100%
	0.4x LD50	10.85	0.67	100%
	0.6x LD50	6.94	0.22	64%*
	0.75x LD50	4.77	0.22	44%*
240 hours	Control	12.09	1.31	100%
	0.4x LD50	14.98	1.43	124%
	0.6x LD50	8.27	0.17	68%
	0.75x LD50	7.33	0.25	61%
600 hours	Control	10.96	0.46	100%
	0.4x LD50	9.50	0.37	87%
	0.6x LD50	9.05	0.33	83%
	0.75x LD50	8.24	0.21	75%*

*****Significant changes in enzyme activities ($p<0.05$).

Supplemental Table 3: Sarin-Induced Inhibition of Erythrocyte AChE and Plasma BuChE.

Time Point	Sarin Dose	AChE Activity (nmoles/min/µL)			BuChE Activity (nmoles/min/µL)		
		Average	St. Error	% Control	Average	St. Error	% Control
2 hours	Control	0.48	0.04	100%	0.59	0.03	100%
	0.4x LD50	0.19	0.02	39%*	0.36	0.03	61%*
	0.6x LD50	0.11	0.01	22%*	0.26	0.03	43%*
	0.75x LD50	0.15	0.01	31%*	0.16	0.03	27%*
6 hours	Control	0.35	0.02	100%	0.43	0.05	100%
	0.4x LD50	0.18	0.03	52%*	0.41	0.05	96%
	0.6x LD50	0.11	0.02	31%*	0.42	0.02	98%
	0.75x LD50	0.09	0.01	25%*	0.41	0.02	96%
12 hour	Control	0.44	0.03	100%	0.69	0.03	100%
	0.4x LD50	0.13	0.01	29%*	0.62	0.03	90%
	0.6x LD50	0.14	0.02	32%*	0.61	0.04	89%
	0.75x LD50	0.15	0.01	34%*	0.60	0.01	88%
48 hours	Control	0.28	0.01	100%	0.66	0.05	100%
	0.4x LD50	0.24	0.01	85%	0.60	0.03	91%
	0.6x LD50	0.21	0.02	76%	0.47	0.04	71%
	0.75x LD50	0.24	0.02	85%	0.50	0.02	76%
120 hours	Control	0.32	0.02	100%	0.65	0.04	100%
	0.4x LD50	0.26	0.02	82%	0.55	0.04	86%
	0.6x LD50	0.22	0.02	68%*	0.53	0.03	82%
	0.75x LD50	0.22	0.02	69%*	0.50	0.01	77%
600 hours	Control	0.29	0.02	100%	0.64	0.04	100%
	0.4x LD50	0.24	0.03	83%	0.70	0.02	110%
	0.6x LD50	0.27	0.02	93%	0.65	0.02	102%
	0.75x LD50	0.32	0.01	111%	0.58	0.02	91%

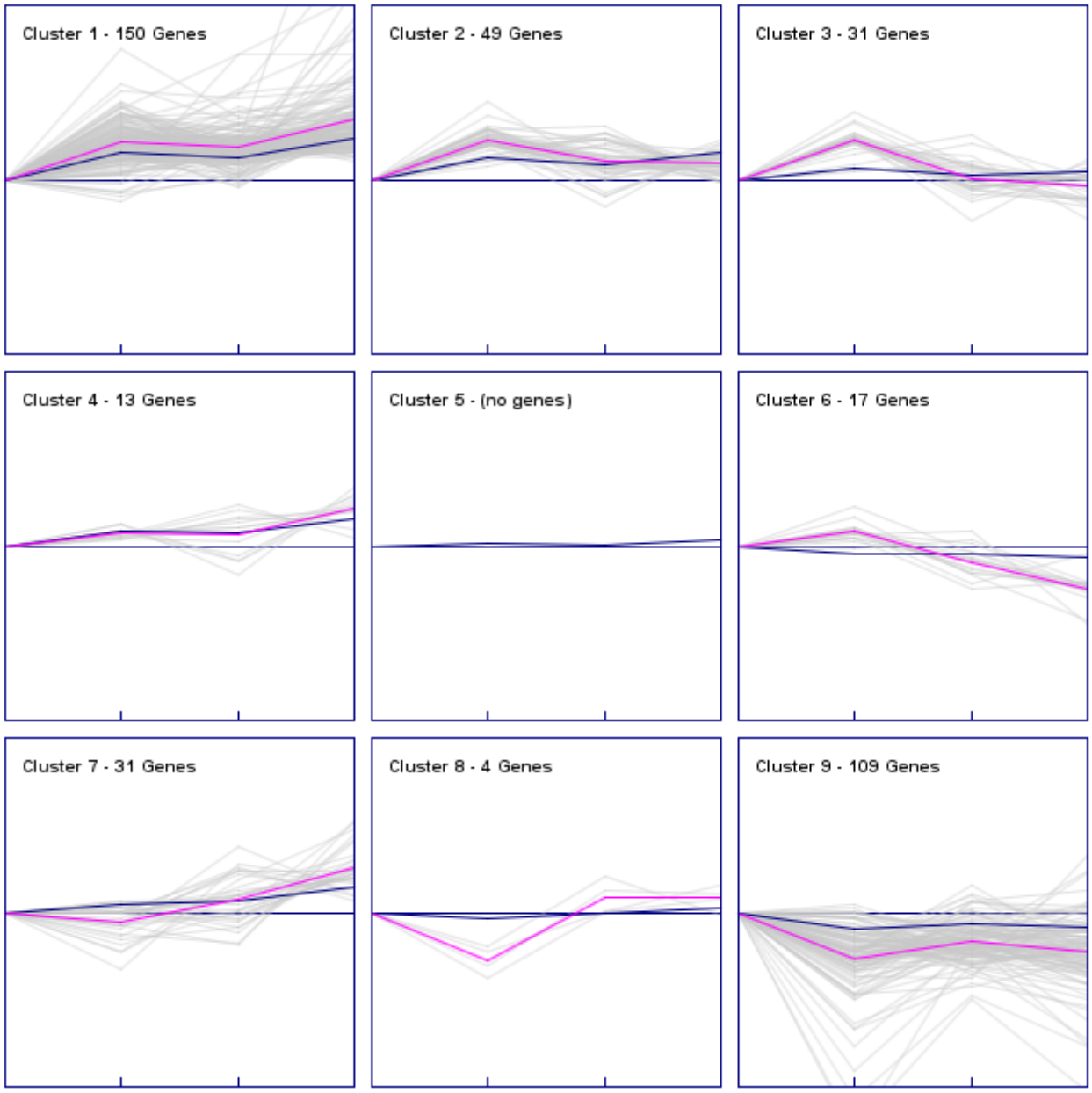
*Significant changes in enzyme activities ($p<0.05$).

Supplemental Table 4: Sarin-Induced Hippocampal Differential Gene Expression.

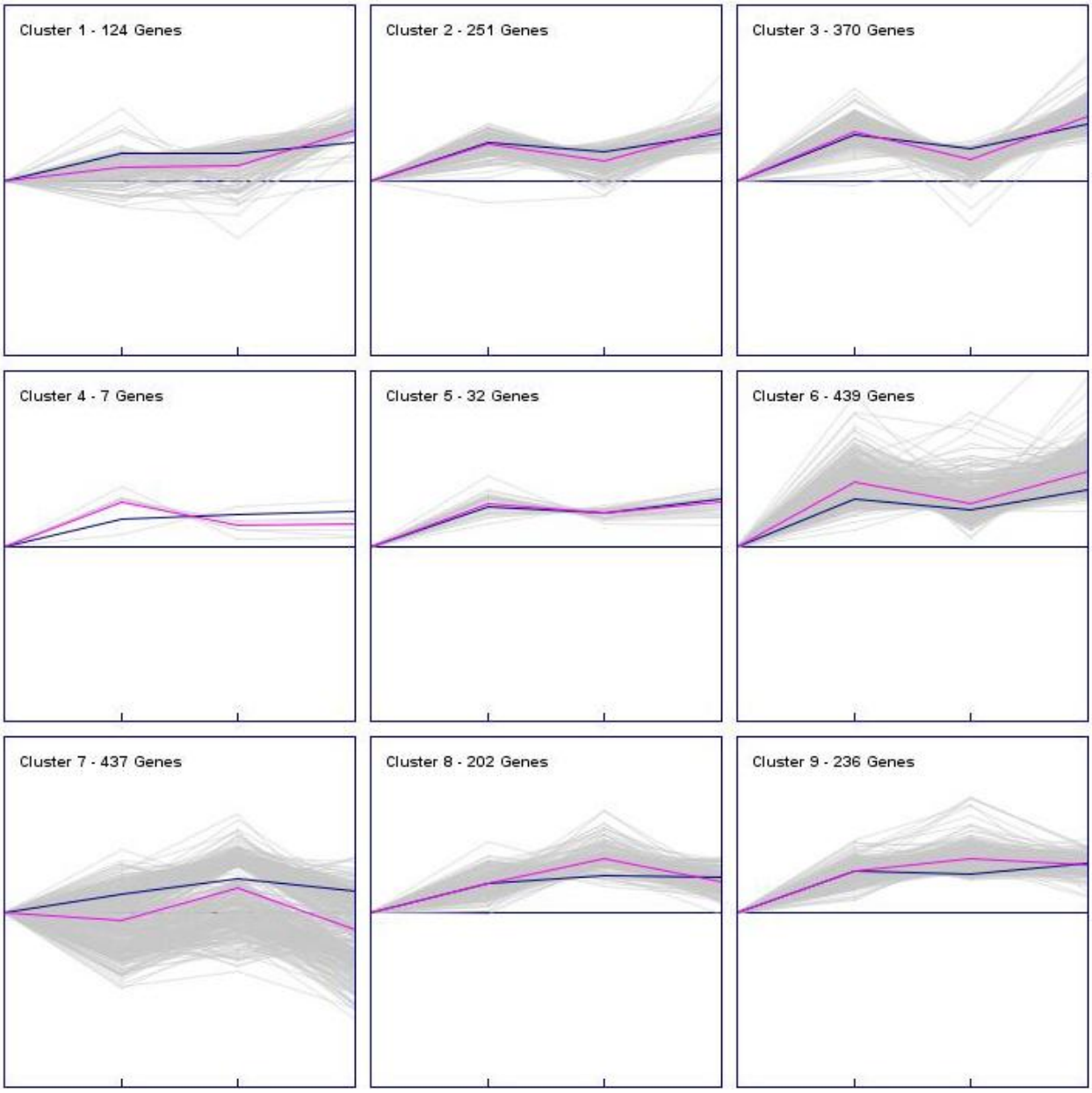
Time after Sarin Treatment (Hr)	2	6	12	24	48	120	240	600
Up-regulated Genes	275	1866	469	372	552	836	73	1684
Down-regulation Genes	129	231	853	248	292	47	457	78
Total Number of Differentially Expressed Genes	404	2097	1322	620	844	883	530	1762

Supplemental Figure 1: Self-Organizing Maps of Differentially Expressed Genes

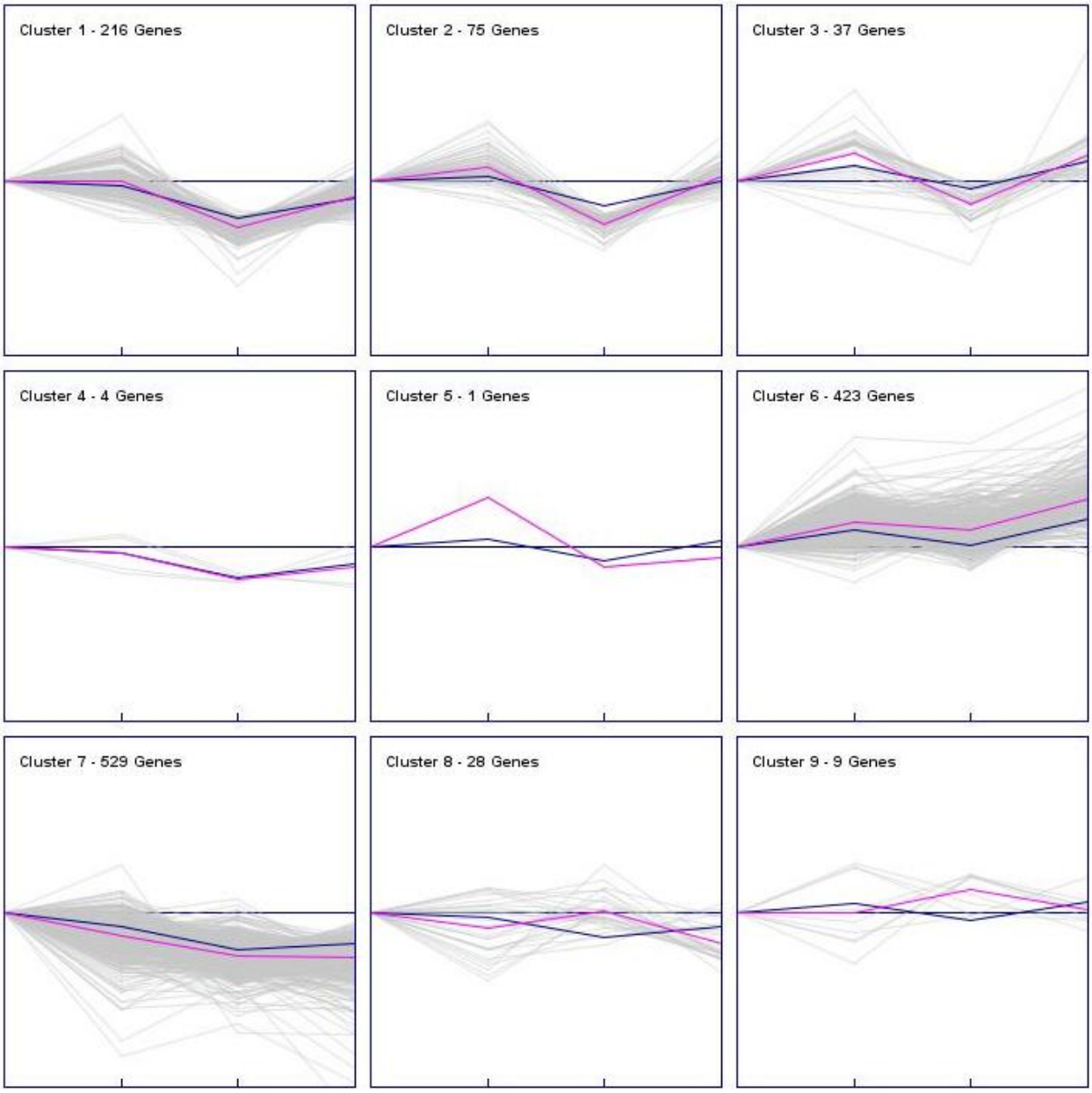
A. 2 Hours



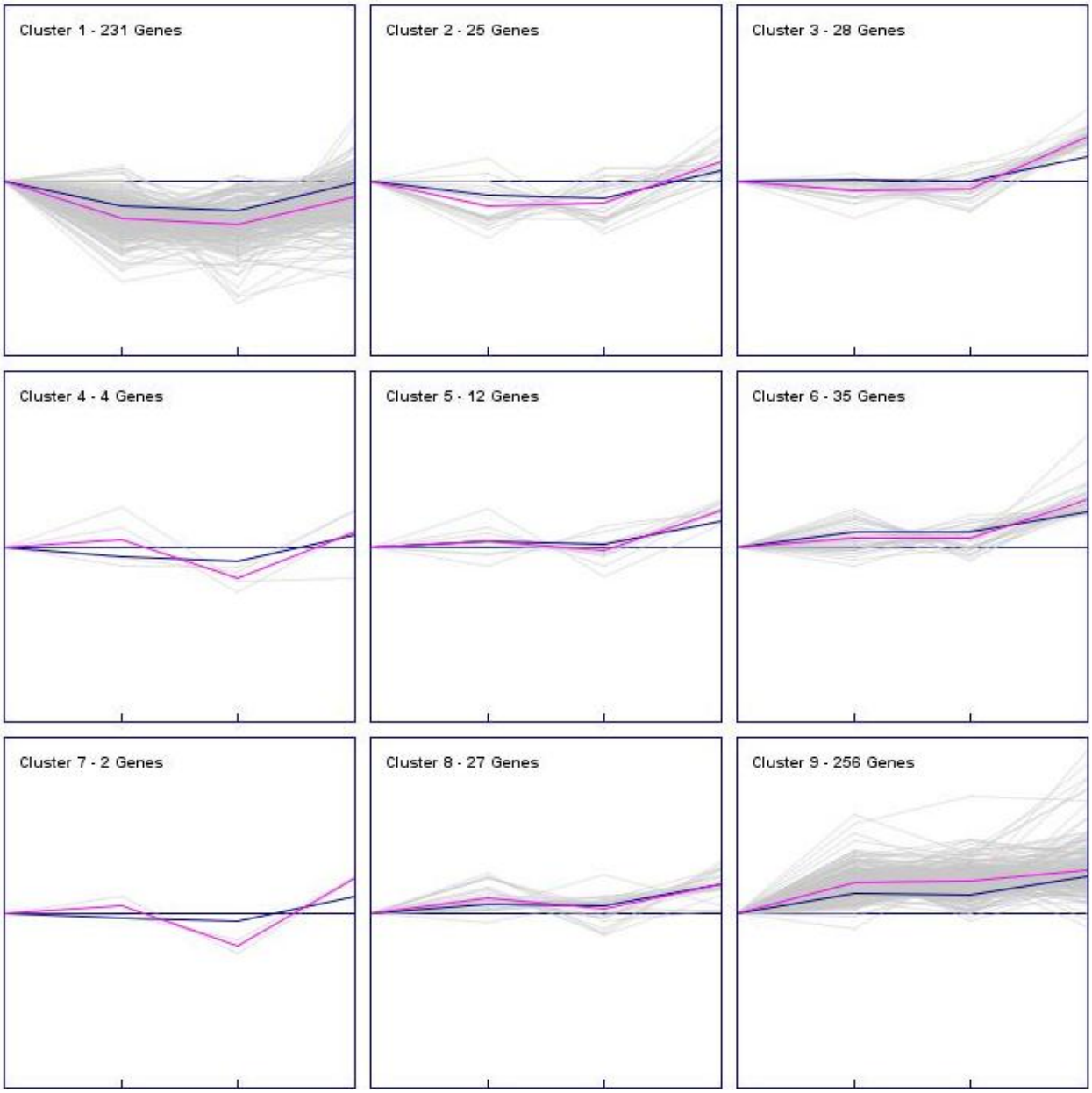
B. 6 Hours



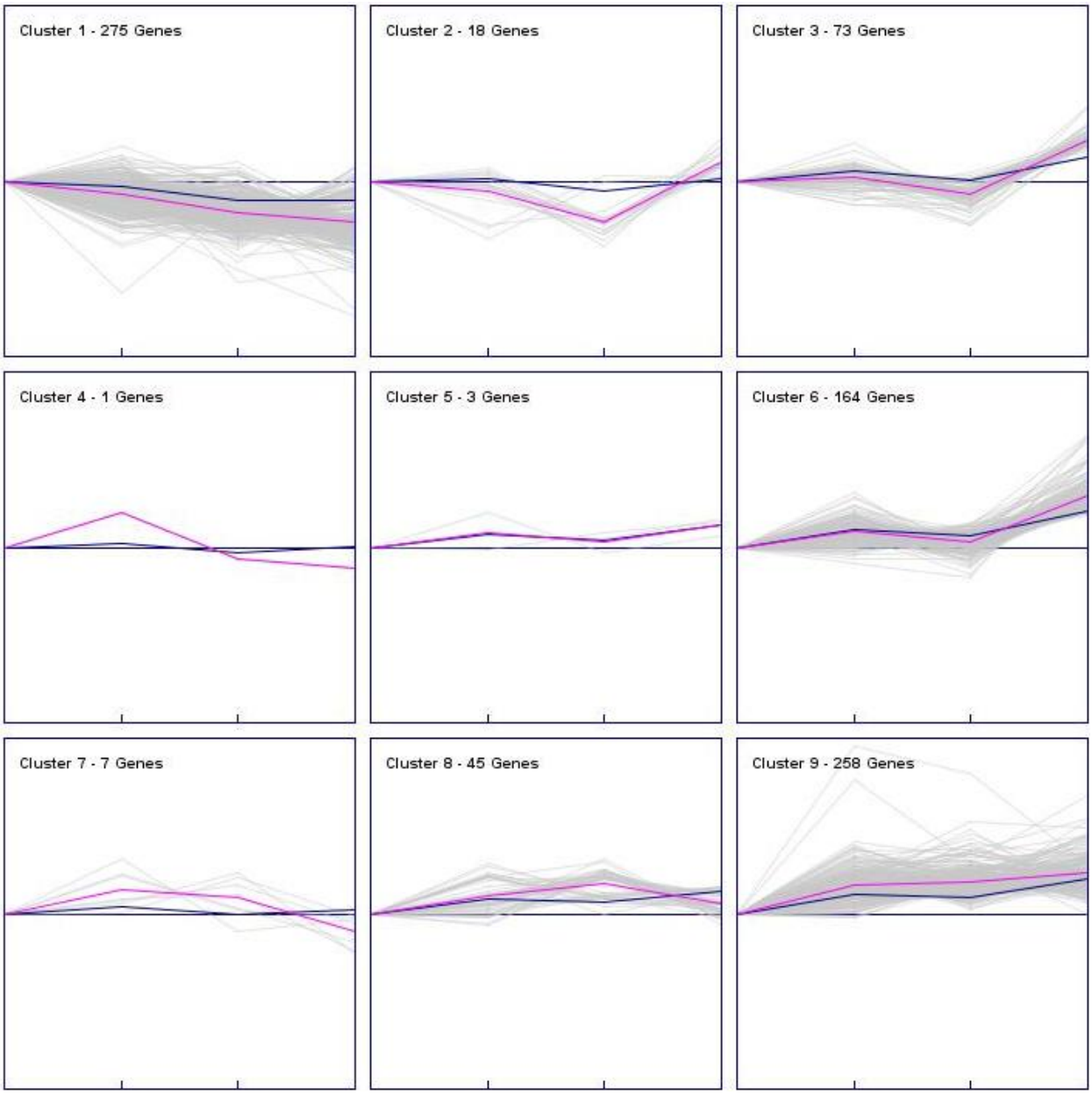
C. 12 Hours



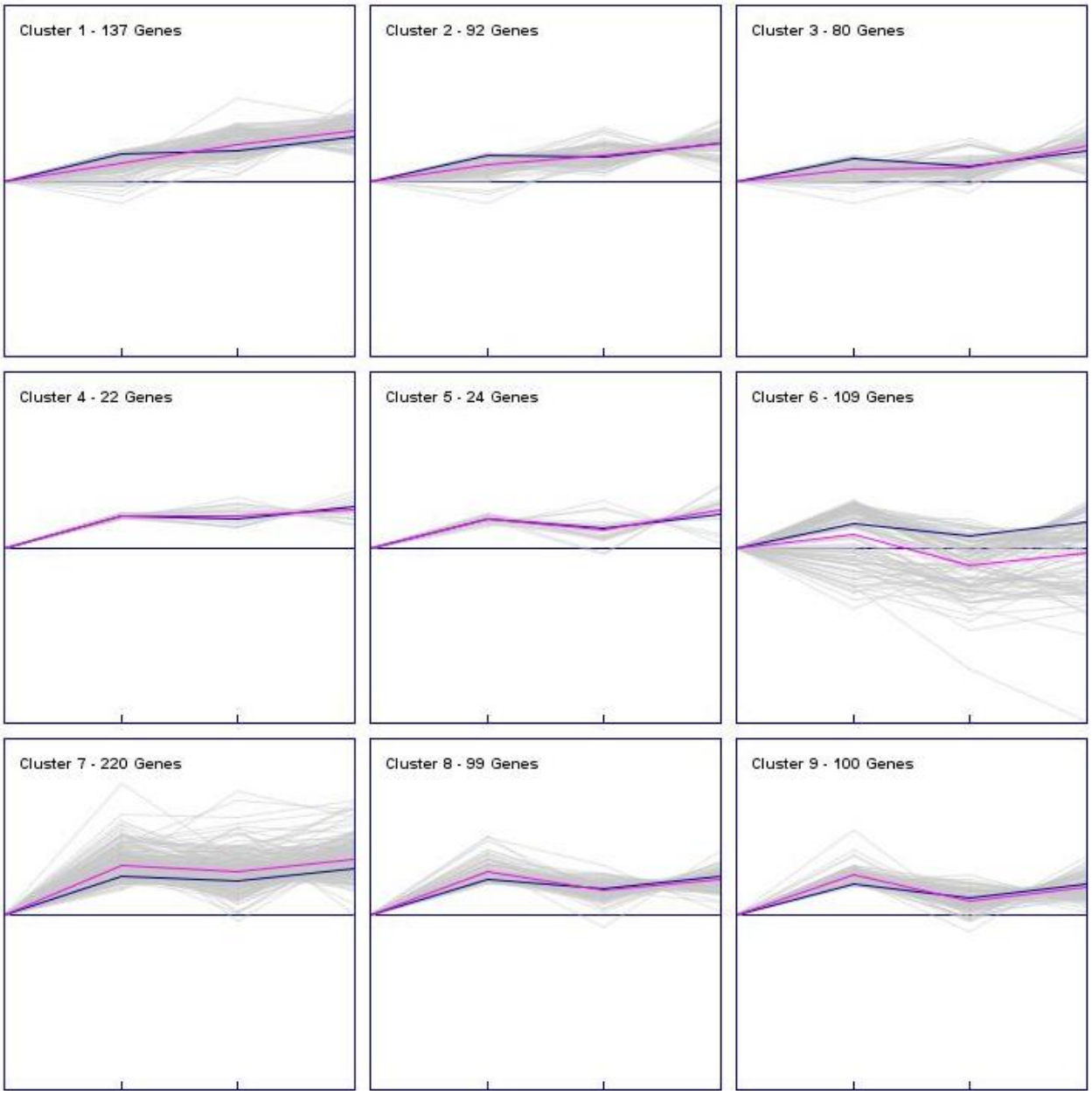
D. 24 Hours



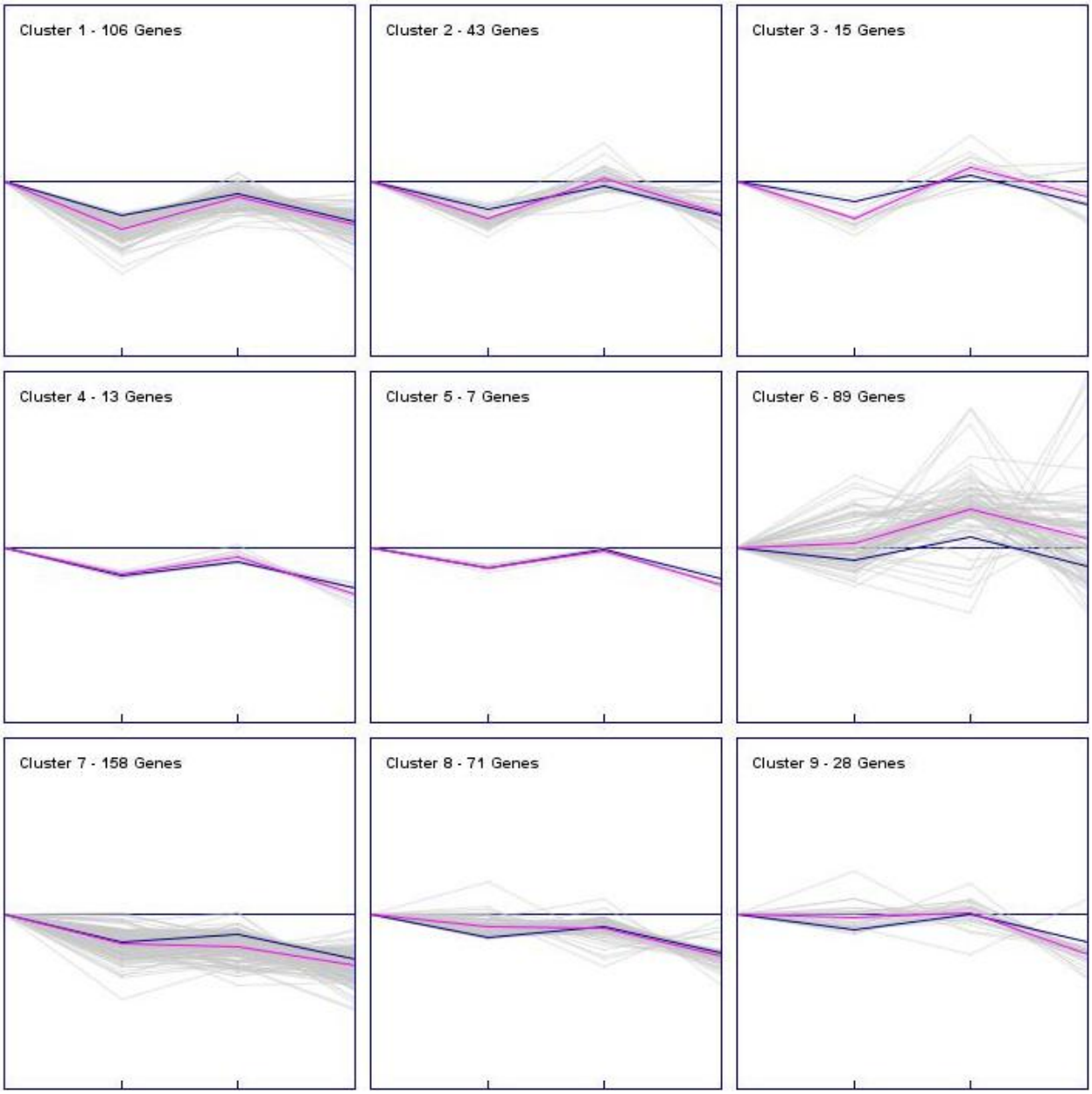
E. 2 Days



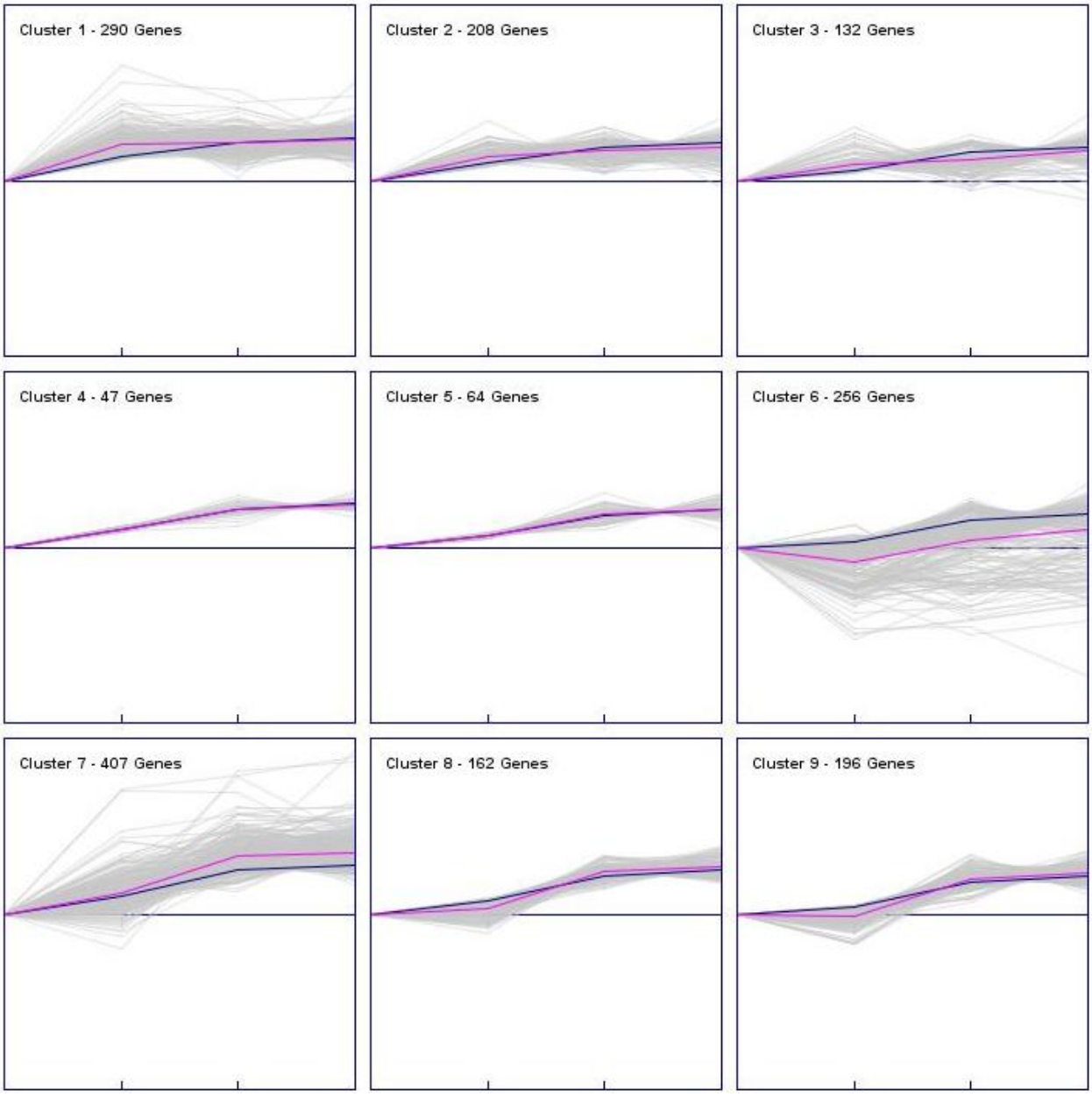
F. 5 Days



G. 10 Days



H. 25 Days



Supplemental Table 5: Complete Lists of Pathways Showing Perturbation (Significant Enrichment of Differentially Expressed Genes)

A. 2 Hours

Biological Process and Pathway	Count	Change Direction
mRNA metabolic process	17	Up-regulation
mRNA processing	14	Up-regulation
regulation of mRNA stability	3	Up-regulation
RNA splicing	10	Up-regulation
Spliceosome	9	Up-regulation
ribonucleoprotein complex	15	Up-regulation
programmed cell death	13	Up-regulation
positive regulation of programmed cell death	8	Up-regulation
regulation of programmed cell death	16	Up-regulation
regulation of cell proliferation	16	Up-regulation
positive regulation of cell proliferation	9	Up-regulation
negative regulation of cell proliferation	8	Up-regulation
negative regulation of cell differentiation	8	Up-regulation
amino acid transport	6	Up-regulation
vasculature development	9	Up-regulation
endoplasmic reticulum	22	Up-regulation
ubl conjugation	19	Up-regulation
lysosome	4	Down-regulation
lytic vacuole	4	Down-regulation
ribosome	6	Down-regulation
ribonucleoprotein complex	7	Down-regulation
ribosomal protein	6	Down-regulation
oxidative phosphorylation	4	Down-regulation
posttranscriptional regulation of gene expression	8	N/A*

*N/A – Not applicable, result obtained by combining up- and down-regulated genes.

B. 6 Hours

Biological Process and Pathway	Count	Change Direction
transcription	218	Up-regulation
transcription regulation	177	Up-regulation
posttranscriptional regulation of gene expression	31	Up-regulation
mRNA processing	54	Up-regulation
RNA splicing	37	Up-regulation
translation	45	Up-regulation
regulation of translation	22	Up-regulation
regulation of eIF4e and p70 S6 Kinase	8	Up-regulation
negative regulation of translation	8	Up-regulation
protein localization	107	Up-regulation
establishment of protein localization	87	Up-regulation
maintenance of protein location in cell	7	Up-regulation
protein localization in mitochondrion	6	Up-regulation
protein transport	86	Up-regulation
intracellular protein transport	46	Up-regulation
vesicle-mediated transport	61	Up-regulation
protein import	14	Up-regulation
protein targeting	25	Up-regulation
protein targeting to mitochondrion	6	Up-regulation
negative regulation of protein complex disassembly	8	Up-regulation
cellular protein catabolic process	100	Up-regulation
ubiquitin-dependent protein catabolic process	35	Up-regulation
generation of precursor metabolites and energy	33	Up-regulation
mitochondrion organization	16	Up-regulation
electron transport chain	17	Up-regulation
peroxisome organization	6	Up-regulation
phospholipid biosynthetic process	14	Up-regulation
cytoskeleton organization	40	Up-regulation
regulation of cytoskeleton organization	18	Up-regulation
microtubule-based process	29	Up-regulation
regulation of microtubule cytoskeleton organization	9	Up-regulation
regulation of microtubule depolymerization	5	Up-regulation
negative regulation of microtubule depolymerization	5	Up-regulation
cellular response to stress	51	Up-regulation
response to DNA damage stimulus	37	Up-regulation
DNA repair	30	Up-regulation
cellular response to insulin stimulus	9	Up-regulation
Signaling Pathway from G-Protein Families	10	Up-regulation
transmembrane receptor protein tyrosine kinase signaling pathway	32	Up-regulation
cell cycle	69	Up-regulation
cell motion	44	Up-regulation
cell migration	32	Up-regulation
neuron projection development	34	Up-regulation
neuron projection morphogenesis	25	Up-regulation
cell morphogenesis involved in neuron differentiation	24	Up-regulation
axonogenesis	23	Up-regulation
dendrite development	11	Up-regulation
synaptogenesis	6	Up-regulation
regulation of long-term neuronal synaptic plasticity	9	Up-regulation
synaptic transmission	31	Up-regulation

adult locomotory behavior	11	Up-regulation
learning or memory	15	Up-regulation
learning	12	Up-regulation
transmission of nerve impulse	36	Up-regulation
regulation of synaptic transmission	17	Up-regulation
regulation of neuronal synaptic plasticity	11	Up-regulation
nerve-nerve synaptic transmission	9	Up-regulation
synaptic transmission, glutamatergic	5	Up-regulation
glycerophospholipid biosynthetic process	9	Up-regulation
phosphatidylcholine biosynthetic process	4	Up-regulation
regulation of sequestering of triglyceride	3	Up-regulation
potassium ion transport	22	Up-regulation
regulation of membrane potential	19	Up-regulation
spliceosome	5	Down-regulation
mitochondrion	23	Down-regulation
endoplasmic reticulum	19	Down-regulation
lysosome	6	Down-regulation
lytic vacuole	6	Down-regulation
lipid synthesis	5	Down-regulation
synaptosome	3	Down-regulation
gene silencing	12	N/A*
mRNA splicing	41	N/A*
mRNA transport	12	N/A*
ribosome	35	N/A*
ribosomal protein	33	N/A*
protein biosynthesis	26	N/A*
translation regulation	17	N/A*
endomembrane system	71	N/A*
Golgi apparatus	83	N/A*
ER-Golgi transport	14	N/A*
vesicle	65	N/A*
vesicle-mediated transport	67	N/A*
chaperone	24	N/A*
ubl conjugation pathway	88	N/A*
ubiquitin mediated proteolysis	23	N/A*
proteasome complex	18	N/A*
mitochondrion	165	N/A*
electron transport	16	N/A*
cytoskeleton	133	N/A*
regulation of actin cytoskeleton	29	N/A*
microtubule cytoskeleton	59	N/A*
microtubule	31	N/A*
microtubule cytoskeleton organization	21	N/A*
dynein complex	10	N/A*
DNA damage	28	N/A*
apoptosis	46	N/A*
cell-cell signaling	39	N/A*
Neurotrophin signaling pathway	23	N/A*
ErbB signaling pathway	18	N/A*
Insulin signaling pathway	29	N/A*
MAPK signaling pathway	41	N/A*
mTOR signaling pathway	12	N/A*
neurogenesis	22	N/A*

focal adhesion	33	N/A*
adherens junction	14	N/A*
neuron projection	44	N/A*
axon guidance	21	N/A*
dendrite	21	N/A*
synapse	54	N/A*
synapse organization	10	N/A*
postsynaptic membrane	24	N/A*
postsynaptic density	15	N/A*
presynaptic membrane	9	N/A*
Alzheimer's disease	28	N/A*
Glioma	14	N/A*
Long-term potentiation	21	N/A*
Long-term depression	14	N/A*

*N/A – Not applicable, result obtained by combining up- and down-regulated genes.

C. 12 Hours

Biological Process and Pathway	Count	Change Direction
mRNA metabolic process	20	Up-regulation
mRNA processing	18	Up-regulation
RNA splicing	17	Up-regulation
establishment of RNA localization	7	Up-regulation
mRNA transport	6	Up-regulation
ribonucleoprotein complex	28	Up-regulation
regulation of translation	7	Up-regulation
cellular protein catabolic process	27	Up-regulation
proteasome complex	7	Up-regulation
protein catabolic process	29	Up-regulation
ubl conjugation pathway	20	Up-regulation
ubiquitin-dependent protein catabolic process	14	Up-regulation
cytoskeletal part	25	Up-regulation
regulation of actin cytoskeleton	11	Up-regulation
actin cytoskeleton organization	9	Up-regulation
enzyme linked receptor protein signaling pathway	15	Up-regulation
MAPK signaling pathway	16	Up-regulation
transforming growth factor beta receptor signaling pathway	5	Up-regulation
neuron projection	10	Up-regulation
dendrite	8	Up-regulation
dendritic spine	4	Up-regulation
dendritic shaft	3	Up-regulation
synapse	20	Up-regulation
synapse part	14	Up-regulation
presynaptic membrane	6	Up-regulation
postsynaptic membrane	10	Up-regulation
postsynaptic density	8	Up-regulation
neurogenesis	8	Up-regulation
regulation of neuron differentiation	6	Up-regulation
learning or memory	8	Up-regulation
learning	5	Up-regulation
memory	4	Up-regulation
synaptic transmission	12	Up-regulation
regulation of synaptic transmission	9	Up-regulation
regulation of synaptic plasticity	5	Up-regulation
neurotransmitter secretion	5	Up-regulation
vasculature development	13	Up-regulation
respiratory chain	5	Up-regulation
cell-cell signaling	14	Up-regulation
transmembrane receptor protein tyrosine kinase signaling pathway	10	Up-regulation
wnt signaling pathway	7	Up-regulation
regulation of transcription	105	Down-regulation
negative regulation of transcription, DNA-dependent	20	Down-regulation
transcription corepressor activity	7	Down-regulation
RNA processing	26	Down-regulation
mRNA processing	19	Down-regulation
RNA splicing	16	Down-regulation
negative regulation of RNA metabolic process	20	Down-regulation
cytoplasmic vesicle	31	Down-regulation
protein transport	44	Down-regulation

endoplasmic reticulum	43	Down-regulation
Golgi apparatus	41	Down-regulation
trans-Golgi network	9	Down-regulation
vesicle	32	Down-regulation
regulation of protein ubiquitination	5	Down-regulation
chromatin modification	17	Down-regulation
histone modification	10	Down-regulation
H4/H2A histone acetyltransferase complex	5	Down-regulation
histone H2A acetylation	4	Down-regulation
histone H4 acetylation	4	Down-regulation
Glutathione S-transferase, C-terminal	7	Down-regulation
Glutathione S-transferase, Mu class	4	Down-regulation
Glutathione S-transferase, N-terminal	7	Down-regulation
Glutathione S-transferase, C-terminal-like	5	Down-regulation
Glutathione S-transferase/chloride channel, C-terminal	8	Down-regulation
Metabolism of xenobiotics by cytochrome P450	8	Down-regulation
mitochondrion	71	Down-regulation
mitochondrial lumen	14	Down-regulation
mitochondrial matrix	14	Down-regulation
microtubule-based movement	8	Down-regulation
endocytosis	13	Down-regulation
regulation of endocytosis	6	Down-regulation
response to nutrient levels	11	Down-regulation
glucose metabolic process	11	Down-regulation
carbohydrate biosynthetic process	8	Down-regulation
Arginine and proline metabolism	7	Down-regulation
response to temperature stimulus	7	Down-regulation
cell cycle	36	Down-regulation
regulation of growth	19	Down-regulation
positive regulation of cell differentiation	14	Down-regulation
cerebral cortex neuron differentiation	3	Down-regulation
potassium channel complex	8	Down-regulation
voltage-gated potassium channel complex	8	Down-regulation
ribonucleoprotein complex	30	Down-regulation
membrane lipid metabolic process	7	Down-regulation
membrane lipid catabolic process	4	Down-regulation
sphingolipid catabolic process	4	Down-regulation
androgen receptor signaling pathway	3	Down-regulation
intracellular receptor-mediated signaling pathway	6	Down-regulation
steroid hormone receptor signaling pathway	5	Down-regulation
vesicle-mediated transport	41	N/A*
membrane invagination	20	N/A*
histone acetyltransferase complex	8	N/A*
regulation of cell cycle	20	N/A*
cell cycle arrest	8	N/A*
regulation of neurogenesis	14	N/A*
forebrain neuron differentiation	4	N/A*

*N/A – Not applicable, result obtained by combining up- and down-regulated genes.

D. 24 Hours

Biological Process and Pathway	Count	Change Direction
positive regulation of gene expression	17	Up-regulation
negative regulation of gene expression	14	Up-regulation
positive regulation of transcription	17	Up-regulation
negative regulation of transcription	13	Up-regulation
regulation of transcription, DNA-dependent	35	Up-regulation
positive regulation of transcription, DNA-dependent	16	Up-regulation
negative regulation of transcription, DNA-dependent	12	Up-regulation
regulation of transcription from RNA polymerase II promoter	21	Up-regulation
positive regulation of transcription from RNA polymerase II promoter	13	Up-regulation
negative regulation of transcription from RNA polymerase II promoter	11	Up-regulation
positive regulation of RNA metabolic process	16	Up-regulation
cell-cell signaling	13	Up-regulation
MAPK signaling pathway	12	Up-regulation
vasculature development	9	Up-regulation
blood vessel morphogenesis	8	Up-regulation
wound healing	8	Up-regulation
angiogenesis	6	Up-regulation
regulation of cell proliferation	17	Up-regulation
positive regulation of cell proliferation	12	Up-regulation
regulation of growth	10	Up-regulation
negative regulation of cell differentiation	9	Up-regulation
neuron differentiation	14	Up-regulation
regulation of neurogenesis	8	Up-regulation
regulation of cell death	17	Up-regulation
regulation of apoptosis	17	Up-regulation
positive regulation of cell death	10	Up-regulation
positive regulation of apoptosis	10	Up-regulation
axon	5	Up-regulation
regulation of neuron projection development	4	Up-regulation
synapse	13	Up-regulation
synapse part	9	Up-regulation
postsynaptic density	4	Up-regulation
transmission of nerve impulse	9	Up-regulation
regulation of synaptic transmission	6	Up-regulation
regulation of excitatory postsynaptic membrane potential	3	Up-regulation
regulation of neuronal synaptic plasticity	3	Up-regulation
ion transport	15	Up-regulation
vitamin A metabolic process	5	Up-regulation
response to insulin stimulus	4	Up-regulation
response to dietary excess	3	Up-regulation
regulation of hormone levels	9	Up-regulation
actin cytoskeleton	8	Up-regulation
regulation of membrane potential	6	Up-regulation
regulation of calcium ion transport	4	Up-regulation
positive regulation of transcription from RNA polymerase II promoter	9	Down-regulation
posttranscriptional regulation of gene expression	7	Down-regulation
translation regulation	4	Down-regulation

Golgi apparatus	20	Down-regulation
trans-Golgi network	4	Down-regulation
regulation of protein localization	5	Down-regulation
cytoplasmic membrane-bounded vesicle	12	Down-regulation
cytoplasmic vesicle	18	Down-regulation
vesicle	18	Down-regulation
vesicle-mediated transport	11	Down-regulation
mitochondrial envelope	11	Down-regulation
mitochondrial part	13	Down-regulation
mitochondrial inner membrane	9	Down-regulation
generation of precursor metabolites and energy	8	Down-regulation
MAPK signaling pathway	8	Down-regulation
negative regulation of MAP kinase activity	3	Down-regulation
actin filament bundle	3	Down-regulation
regulation of angiogenesis	4	Down-regulation
myelination	3	Down-regulation
synaptosome	5	Down-regulation
regulation of membrane potential	6	Down-regulation
ion transport	16	Down-regulation
regulation of cell adhesion	6	Down-regulation
growth cone	4	Down-regulation
site of polarized growth	4	Down-regulation
negative regulation of cell adhesion	3	Down-regulation
regulation of protein transport	6	N/A*
protein kinase cascade	14	N/A*
regulation of protein kinase cascade	10	N/A*
response to wounding	18	N/A*
cell migration	15	N/A*
cell motility	15	N/A*
axon ensheathment	6	N/A*
dendrite	8	N/A*
dendritic spine	4	N/A*
regulation of dendrite development	3	N/A*
regulation of action potential	6	N/A*
regulation of action potential in neuron	6	N/A*
regulation of postsynaptic membrane potential	4	N/A*
regulation of long-term neuronal synaptic plasticity	4	N/A*
sodium ion transport	10	N/A*
regulation of ion transport	6	N/A*

*N/A – Not applicable, result obtained by combining up- and down-regulated genes.

E. 48 hours

Biological Process and Pathway	Count	Change Direction
regulation of transcription, DNA-dependent	46	Up-regulation
negative regulation of transcription	15	Up-regulation
RNA splicing	11	Up-regulation
spliceosome	7	Up-regulation
protein folding	8	Up-regulation
proteolysis	39	Up-regulation
proteolysis involved in cellular protein catabolic process	28	Up-regulation
ubl conjugation pathway	24	Up-regulation
ubiquitin-dependent protein catabolic process	11	Up-regulation
proteasome complex	6	Up-regulation
proteasome	5	Up-regulation
nuclear envelope-endoplasmic reticulum network	10	Up-regulation
endoplasmic reticulum part	13	Up-regulation
endoplasmic reticulum membrane	9	Up-regulation
cholesterol biosynthetic process	7	Up-regulation
lipid synthesis	14	Up-regulation
steroid biosynthetic process	9	Up-regulation
sterol biosynthetic process	8	Up-regulation
cell-cell signaling	18	Up-regulation
MAPK signaling pathway	12	Up-regulation
TGF-beta signaling pathway	7	Up-regulation
Neurotrophin signaling pathway	8	Up-regulation
response to hormone stimulus	9	Up-regulation
response to peptide hormone stimulus	7	Up-regulation
response to insulin stimulus	5	Up-regulation
regulation of centrosome duplication	3	Up-regulation
cell morphogenesis involved in differentiation	15	Up-regulation
cell morphogenesis involved in neuron differentiation	14	Up-regulation
regulation of cell growth	7	Up-regulation
positive regulation of cell growth	4	Up-regulation
regulation of neurogenesis	10	Up-regulation
forebrain development	10	Up-regulation
cell proliferation in forebrain	3	Up-regulation
hindbrain development	5	Up-regulation
neuron development	20	Up-regulation
neuron differentiation	23	Up-regulation
regulation of neuron differentiation	10	Up-regulation
neuron maturation	3	Up-regulation
regulation of cell size	9	Up-regulation
positive regulation of cell size	4	Up-regulation
multicellular organismal response to stress	5	Up-regulation
regulation of cell death	23	Up-regulation
regulation of apoptosis	23	Up-regulation
regulation of neuron apoptosis	8	Up-regulation
negative regulation of neuron apoptosis	7	Up-regulation
cell projection organization	19	Up-regulation
regulation of cell projection organization	6	Up-regulation
neuron projection development	17	Up-regulation
regulation of neuron projection development	6	Up-regulation
neuron projection morphogenesis	14	Up-regulation

microtubule organizing center	10	Up-regulation
regulation of cytoskeleton organization	7	Up-regulation
axonogenesis	13	Up-regulation
axon guidance	9	Up-regulation
regulation of axonogenesis	5	Up-regulation
positive regulation of axonogenesis	3	Up-regulation
regulation of axon extension	3	Up-regulation
dendrite development	6	Up-regulation
synapse	17	Up-regulation
synapse part	11	Up-regulation
transmission of nerve impulse	14	Up-regulation
regulation of transmission of nerve impulse	9	Up-regulation
positive regulation of transmission of nerve impulse	3	Up-regulation
synaptic transmission	11	Up-regulation
regulation of synaptic transmission	8	Up-regulation
positive regulation of synaptic transmission	3	Up-regulation
regulation of synaptic plasticity	6	Up-regulation
Long-term potentiation	6	Up-regulation
learning or memory	8	Up-regulation
memory	6	Up-regulation
fear response	4	Up-regulation
sensory perception of pain	5	Up-regulation
ion channel activity	16	Up-regulation
calcium channel activity	6	Up-regulation
calcium-activated potassium channel activity	3	Up-regulation
regulation of microtubule-based process	7	Up-regulation
regulation of microtubule cytoskeleton organization	5	Up-regulation
transcription cofactor activity	7	Down-regulation
transcription corepressor activity	4	Down-regulation
protein localization	20	Down-regulation
protein transport	17	Down-regulation
endoplasmic reticulum lumen	4	Down-regulation
Golgi apparatus	18	Down-regulation
membrane lipid metabolic process	4	Down-regulation
sphingolipid metabolic process	4	Down-regulation
glycolipid metabolic process	3	Down-regulation
carbohydrate biosynthetic process	8	Down-regulation
glucose metabolic process	7	Down-regulation
activation of caspase activity by cytochrome c	2	Down-regulation
calcium ion homeostasis	6	Down-regulation
cellular calcium ion homeostasis	6	Down-regulation
potassium ion transport	6	Down-regulation
regulation of membrane potential	5	Down-regulation
synaptic vesicle	5	Down-regulation
regulation of transcription from RNA polymerase II promoter	35	N/A*
transcription repressor activity	16	N/A*
trans-Golgi network	6	N/A*
positive regulation of cell death	17	N/A*
positive regulation of axon extension	3	N/A*
response to light stimulus	9	N/A*

*N/A – Not applicable, result obtained by combining up- and down-regulated genes.

F. 120 hours

Biological Process and Pathway	Count	Change Direction
transcription	87	Up-regulation
regulation of transcription	103	Up-regulation
mRNA metabolic process	19	Up-regulation
posttranscriptional regulation of gene expression	12	Up-regulation
spliceosome	10	Up-regulation
establishment of RNA localization	7	Up-regulation
mRNA transport	6	Up-regulation
translation	29	Up-regulation
regulation of translation	9	Up-regulation
negative regulation of translation	4	Up-regulation
translation factor activity, nucleic acid binding	14	Up-regulation
translational initiation	6	Up-regulation
translation initiation factor activity	9	Up-regulation
translation elongation factor activity	5	Up-regulation
ribonucleoprotein complex	36	Up-regulation
ribosome	21	Up-regulation
ribosomal subunit	9	Up-regulation
large ribosomal subunit	6	Up-regulation
ribosomal protein	18	Up-regulation
protein complex assembly	19	Up-regulation
cellular protein complex assembly	12	Up-regulation
regulation of protein complex assembly	7	Up-regulation
protein localization	60	Up-regulation
establishment of protein localization	50	Up-regulation
endomembrane system	37	Up-regulation
cellular protein localization	25	Up-regulation
protein localization in organelle	9	Up-regulation
protein localization in nucleus	6	Up-regulation
protein transport	35	Up-regulation
intracellular protein transport	23	Up-regulation
nucleocytoplasmic transport	8	Up-regulation
protein targeting	12	Up-regulation
protein import	9	Up-regulation
protein import into nucleus	6	Up-regulation
protein catabolic process	31	Up-regulation
cellular protein catabolic process	30	Up-regulation
proteolysis involved in cellular protein catabolic process	30	Up-regulation
ubl conjugation	35	Up-regulation
ubiquitin	6	Up-regulation
ubiquitin-conjugating enzyme/RWD-like	7	Up-regulation
proteasome	6	Up-regulation
proteasome core complex	4	Up-regulation
proteasome, subunit alpha/beta	4	Up-regulation
cytoskeleton organization	20	Up-regulation
regulation of cytoskeleton organization	11	Up-regulation
regulation of microtubule cytoskeleton organization	8	Up-regulation
regulation of microtubule-based process	8	Up-regulation
regulation of microtubule polymerization	5	Up-regulation
microtubule polymerization	3	Up-regulation
cell motion	25	Up-regulation

cell motility	20	Up-regulation
localization of cell	20	Up-regulation
cell migration	18	Up-regulation
cell-cell signaling	25	Up-regulation
intracellular signaling cascade	46	Up-regulation
MAPK signaling pathway	21	Up-regulation
small GTPase mediated signal transduction	19	Up-regulation
transmembrane receptor protein tyrosine kinase signaling pathway	13	Up-regulation
transforming growth factor beta receptor signaling pathway	6	Up-regulation
chaperone	14	Up-regulation
heat shock protein binding	7	Up-regulation
Heat shock protein DnaJ, conserved site	7	Up-regulation
unfolded protein binding	8	Up-regulation
unfolded protein response	5	Up-regulation
response to endoplasmic reticulum stress	4	Up-regulation
ER overload response	3	Up-regulation
triglyceride biosynthetic process	3	Up-regulation
mitochondrion	66	Up-regulation
respiratory chain	9	Up-regulation
oxidative phosphorylation	5	Up-regulation
ion transport	40	Up-regulation
ion homeostasis	19	Up-regulation
cellular ion homeostasis	18	Up-regulation
Calcium signaling pathway	15	Up-regulation
calcium ion transport	12	Up-regulation
Gap junction	8	Up-regulation
transepithelial chloride transport	3	Up-regulation
neurogenesis	11	Up-regulation
regulation of growth	17	Up-regulation
regulation of cell growth	9	Up-regulation
regulation of endothelial cell proliferation	4	Up-regulation
mitotic cell cycle	16	Up-regulation
interphase of mitotic cell cycle	6	Up-regulation
cell morphogenesis	19	Up-regulation
cell morphogenesis involved in neuron differentiation	15	Up-regulation
chromatin	12	Up-regulation
chromatin regulator	15	Up-regulation
chromatin modification	16	Up-regulation
chromatin remodeling complex	7	Up-regulation
histone acetylation	5	Up-regulation
axonogenesis	13	Up-regulation
cellular component morphogenesis	21	Up-regulation
cell projection organization	24	Up-regulation
regulation of cell morphogenesis	8	Up-regulation
neuron projection development	15	Up-regulation
neuron projection morphogenesis	15	Up-regulation
axon guidance	14	Up-regulation
dendrite	10	Up-regulation
presynaptic membrane	6	Up-regulation
regulation of membrane potential	11	Up-regulation
membrane depolarization	6	Up-regulation
regulation of postsynaptic membrane potential	6	Up-regulation
regulation of excitatory postsynaptic membrane potential	5	Up-regulation

transmission of nerve impulse	24	Up-regulation
regulation of transmission of nerve impulse	12	Up-regulation
synaptic transmission	20	Up-regulation
regulation of synaptic transmission	10	Up-regulation
Endocytosis	14	Up-regulation
regulation of synaptic plasticity	6	Up-regulation
regulation of neuronal synaptic plasticity	5	Up-regulation
Long-term potentiation	12	Up-regulation
behavior	27	Up-regulation
learning or memory	10	Up-regulation
memory	7	Up-regulation
locomotory behavior	16	Up-regulation
response to light stimulus	8	Up-regulation
visual behavior	6	Up-regulation
visual learning	5	Up-regulation
sensory perception of pain	5	Up-regulation
response to ethanol	4	Up-regulation
Alzheimer's disease	15	Up-regulation
Amyotrophic lateral sclerosis (ALS)	8	Up-regulation
Huntington's disease	14	Up-regulation
Parkinson's disease	11	Up-regulation
aging	7	Up-regulation
response to drug	10	Up-regulation
endoplasmic reticulum	7	Down-regulation
regulation of fatty acid biosynthetic process	2	Down-regulation
regulation of steroid biosynthetic process	2	Down-regulation
negative regulation of cell differentiation	4	Down-regulation
anti-proliferative protein	2	Down-regulation
neutral lipid biosynthetic process	3	N/A*

*N/A – Not applicable, result obtained by combining up- and down-regulated genes.

G. 240 Hours

Biological Process and Pathway	Count	Change Direction
mRNA metabolic process	14	Down-regulation
ncRNA metabolic process	10	Down-regulation
RNA processing	21	Down-regulation
mRNA processing	13	Down-regulation
ncRNA processing	8	Down-regulation
RNA splicing	9	Down-regulation
ribonucleoprotein complex	21	Down-regulation
endomembrane system	20	Down-regulation
endoplasmic reticulum	38	Down-regulation
endoplasmic reticulum part	15	Down-regulation
endoplasmic reticulum membrane	10	Down-regulation
nuclear envelope-endoplasmic reticulum network	10	Down-regulation
ER-Golgi transport	6	Down-regulation
Golgi apparatus	28	Down-regulation
Golgi apparatus part	13	Down-regulation
trans-Golgi network	7	Down-regulation
protein localization	30	Down-regulation
establishment of protein localization	25	Down-regulation
cellular protein localization	12	Down-regulation
protein transport	22	Down-regulation
intracellular protein transport	10	Down-regulation
vesicle-mediated transport	18	Down-regulation
cellular protein catabolic process	19	Down-regulation
proteolysis involved in cellular protein catabolic process	19	Down-regulation
ubl conjugation pathway	18	Down-regulation
ER-associated protein catabolic process	3	Down-regulation
autophagy	5	Down-regulation
autophagy	4	Down-regulation
macroautophagy	3	Down-regulation
lysosome	9	Down-regulation
lipid biosynthetic process	12	Down-regulation
fatty acid biosynthetic process	5	Down-regulation
mitochondrial inner membrane	12	Down-regulation
generation of precursor metabolites and energy	10	Down-regulation
cellular response to nutrient levels	4	Down-regulation
cytoplasmic vesicle	18	Down-regulation
intracellular receptor-mediated signaling pathway	4	Down-regulation
glutathione metabolism	5	Down-regulation
glutathione S-transferase, Mu class	3	Down-regulation
glutathione S-transferase, N-terminal	4	Down-regulation
glutathione S-transferase, C-terminal	5	Down-regulation
glutathione S-transferase/chloride channel, C-terminal	5	Down-regulation
regulation of synaptic transmission	6	Down-regulation
response to cold	3	Down-regulation
proteasomal protein catabolic process	4	N/A*
proteasomal ubiquitin-dependent protein catabolic process	4	N/A*
fatty acid metabolic process	9	N/A*
long-chain fatty acid metabolic process	3	N/A*
learning	5	N/A*
regulation of transmission of nerve impulse	7	N/A*

intracellular receptor-mediated signaling pathway	4	N/A*
regulation of cell proliferation	20	N/A*

*N/A – Not applicable, result obtained by combining up- and down-regulated genes.

H. 600 Hours

Biological Process and Pathway	Count	Change Direction
transcription	193	Up-regulation
regulation of transcription	236	Up-regulation
positive regulation of transcription	54	Up-regulation
negative regulation of transcription	48	Up-regulation
positive regulation of gene expression	54	Up-regulation
negative regulation of gene expression	53	Up-regulation
positive regulation of transcription, DNA-dependent	45	Up-regulation
negative regulation of transcription, DNA-dependent	37	Up-regulation
regulation of transcription from RNA polymerase II promoter	65	Up-regulation
negative regulation of transcription from RNA polymerase II promoter	31	Up-regulation
transcription factor complex	27	Up-regulation
mRNA metabolic process	51	Up-regulation
positive regulation of RNA metabolic process	45	Up-regulation
mRNA processing	43	Up-regulation
mRNA splicing	36	Up-regulation
nuclear mRNA splicing, via spliceosome	8	Up-regulation
spliceosome	20	Up-regulation
posttranscriptional regulation of gene expression	26	Up-regulation
mRNA transport	11	Up-regulation
regulation of translation	21	Up-regulation
negative regulation of translation	8	Up-regulation
regulation of translational initiation	7	Up-regulation
endomembrane system	72	Up-regulation
nuclear envelope-endoplasmic reticulum network	21	Up-regulation
endoplasmic reticulum membrane	19	Up-regulation
response to endoplasmic reticulum stress	6	Up-regulation
ER-nuclear signaling pathway	6	Up-regulation
Golgi apparatus	62	Up-regulation
protein localization	81	Up-regulation
establishment of protein localization	68	Up-regulation
protein transport	66	Up-regulation
vesicle-mediated transport	49	Up-regulation
Golgi vesicle transport	9	Up-regulation
intracellular protein transport	31	Up-regulation
regulation of protein complex assembly	12	Up-regulation
regulation of protein complex disassembly	8	Up-regulation
positive regulation of protein complex assembly	6	Up-regulation
Posttranslational modification, protein turnover, chaperones	20	Up-regulation
Molecular chaperone, heat shock protein, Hsp40, DnaJ	8	Up-regulation
glycoprotein biosynthetic process	16	Up-regulation
protein amino acid glycosylation	14	Up-regulation
cellular protein catabolic process	71	Up-regulation
modification-dependent protein catabolic process	70	Up-regulation
proteolysis involved in cellular protein catabolic process	71	Up-regulation
regulation of cellular protein metabolic process	42	Up-regulation
Ubiquitin mediated proteolysis	24	Up-regulation
ubl conjugation	76	Up-regulation
ubl conjugation pathway	71	Up-regulation
phospholipid metabolic process	21	Up-regulation

organophosphate metabolic process	21	Up-regulation
glycerophospholipid metabolic process	14	Up-regulation
mitochondrion inner membrane	22	Up-regulation
cell death	51	Up-regulation
apoptosis	48	Up-regulation
regulation of apoptosis	64	Up-regulation
positive regulation of apoptosis	31	Up-regulation
negative regulation of apoptosis	31	Up-regulation
regulation of cell death	65	Up-regulation
positive regulation of cell death	31	Up-regulation
negative regulation of cell death	31	Up-regulation
cell projection organization	44	Up-regulation
neuron differentiation	43	Up-regulation
neuron development	38	Up-regulation
neuron projection	36	Up-regulation
neuron projection development	31	Up-regulation
cell projection morphogenesis	27	Up-regulation
neuron projection morphogenesis	25	Up-regulation
cell morphogenesis involved in neuron differentiation	25	Up-regulation
axonogenesis	24	Up-regulation
axon guidance	22	Up-regulation
dendrite	19	Up-regulation
dendrite development	8	Up-regulation
dendritic shaft	5	Up-regulation
postsynaptic density	11	Up-regulation
postsynaptic membrane	17	Up-regulation
presynaptic membrane	10	Up-regulation
synapse	43	Up-regulation
synapse part	29	Up-regulation
synapse organization	11	Up-regulation
synaptogenesis	8	Up-regulation
ionotropic glutamate receptor, AMPA/kainate types	4	Up-regulation
synaptic transmission	28	Up-regulation
regulation of synaptic transmission	19	Up-regulation
neurotransmitter secretion	8	Up-regulation
synaptic vesicle transport	7	Up-regulation
synaptic vesicle targeting	3	Up-regulation
regulation of postsynaptic membrane potential	6	Up-regulation
potassium ion transport	22	Up-regulation
calcium ion transport	16	Up-regulation
calcium-dependent cell-cell adhesion	4	Up-regulation
cell motion	45	Up-regulation
cell migration	29	Up-regulation
neuron migration	11	Up-regulation
telencephalon cell migration	6	Up-regulation
forebrain development	25	Up-regulation
pallium development	10	Up-regulation
cerebral cortex development	8	Up-regulation
forebrain cell migration	6	Up-regulation
cerebral cortex cell migration	6	Up-regulation
cellular response to stress	45	Up-regulation
response to insulin stimulus	10	Up-regulation
cellular response to heat	5	Up-regulation

intracellular signaling cascade	92	Up-regulation
MAPK signaling pathway	43	Up-regulation
Phosphatidylinositol signaling system	13	Up-regulation
Insulin signaling pathway	28	Up-regulation
Wnt signaling pathway	22	Up-regulation
Neurotrophin signaling pathway	25	Up-regulation
GnRH signaling pathway	17	Up-regulation
ErbB signaling pathway	19	Up-regulation
TGF-beta signaling pathway	15	Up-regulation
VEGF signaling pathway	14	Up-regulation
mTOR signaling pathway	11	Up-regulation
NOD-like receptor signaling pathway	11	Up-regulation
Transcription factor CREB and its extracellular signals	7	Up-regulation
Chemokine signaling pathway	23	Up-regulation
B cell receptor signaling pathway	17	Up-regulation
T cell receptor signaling pathway	19	Up-regulation
Toll-like receptor signaling pathway	17	Up-regulation
cytoskeleton	105	Up-regulation
regulation of cytoskeleton organization	15	Up-regulation
microtubule	24	Up-regulation
microtubule organizing center	20	Up-regulation
actin cytoskeleton organization	19	Up-regulation
regulation of actin filament-based process	10	Up-regulation
regulation of actin cytoskeleton organization	9	Up-regulation
actin cytoskeleton reorganization	4	Up-regulation
dynein complex	6	Up-regulation
cell cycle	63	Up-regulation
chromosome organization	44	Up-regulation
chromatin modification	30	Up-regulation
chromatin regulator	23	Up-regulation
mitotic sister chromatid segregation	5	Up-regulation
histone methylation	6	Up-regulation
cell adhesion	61	Up-regulation
cell junction	46	Up-regulation
Adherens junction	13	Up-regulation
cell-cell adhesion	32	Up-regulation
Focal adhesion	34	Up-regulation
Gap junction	12	Up-regulation
blood vessel development	28	Up-regulation
Long-term potentiation	15	Up-regulation
Long-term depression	14	Up-regulation
regulation of synaptic plasticity	13	Up-regulation
regulation of neuronal synaptic plasticity	8	Up-regulation
regulation of long-term neuronal synaptic plasticity	7	Up-regulation
behavior	49	Up-regulation
adult locomotory behavior	11	Up-regulation
Alzheimer's disease	20	Up-regulation
Glioma	16	Up-regulation
learning or memory	17	Up-regulation
learning	10	Up-regulation
cellular protein catabolic process	7	Down-regulation
modification-dependent protein catabolic process	7	Down-regulation
proteolysis involved in cellular protein catabolic process	7	Down-regulation

ubl conjugation pathway	7	Down-regulation
Ubiquitin mediated proteolysis	4	Down-regulation
microtubule	4	Down-regulation
Cell cycle	4	Down-regulation
regulation of protein polymerization	10	N/A*
Chaperone	20	N/A*
protein folding	17	N/A*
negative regulation of neuron apoptosis	9	N/A*
positive regulation of cell projection organization	6	N/A*
microtubule cytoskeleton	52	N/A*
Regulation of actin cytoskeleton	28	N/A*
chromatin organization	35	N/A*
ATP biosynthetic process	13	N/A*
vasculature development	30	N/A*

*N/A – Not applicable, result obtained by combining up- and down-regulated genes.